The role of the complement system in the hemolytic uremic syndrome

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Chapter 1

General introduction and outline of the thesis

Partly based on:

A new era in the diagnosis and treatment of atypical haemolytic uremic syndrome.

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Content

The hemolytic uremic syndrome (HUS) is a rare and severe disease, characterized by the triad hemolytic anemia, thrombocytopenia, and acute renal failure. HUS is characterized histologically by thrombotic microangiopathy (TMA): vascular abnormalities with glomerular endothelial damage, swelling of the endothelium, endothelial detachment of the basement membrane, intima fibrosis, and thrombosis. The most important causes of thrombotic microangiopathies (both HUS and thrombotic thrombocytopenic purpura [TTP]) are shown in Table 1.1.[1-3]

Table 1.1. Causes of thrombotic microangiopathy (hemolytic uremic syndromes and thrombotic thrombocytopenic purpura).

Infectious
Infection with Shiga-like toxin producing *Escherichia coli* (STEC-HUS)
Infection with neuraminidase producing *Streptococcus pneumoniae* (SP-HUS)
Human Immunodeficiency virus (HIV)
Complement dysregulation
Genetic abnormalities in complement (regulating) proteins
Acquired defects (autoantibodies against FH)
Mutations in coagulation genes
Mutations in thrombomodulin (*THBD*), diacylglycerol kinase ε (*DGKE*), or plasminogen (*PLG*)
ADAMTS13 deficiency
Mutations in ADAMTS13
Autoantibodies against ADAMTS13
Clinically associated with
Systemic diseases: SLE, antiphospholipid syndrome, defective cobalamin C metabolism
Medication: ticlopedin, mitomycin, bleomycin, cysplatin, quinine, tacrolimus, cyclosporin, rifampicin, clopidopogrel, bevacizumab, interferon γ
Malignancies: chemotherapy
Viruses: cytomegalovirus, parvovirus
Pregnancy: oral contraceptives, pre-eclampsia, HELLP syndrome
Glomerulopathies: C3 glomerulopathy
Bone marrow transplantation: radiation, medication, graft vs host disease

Abbreviations: ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; FH, complement factor H; HELLP, haemolysis, elevated liver enzymes and low platelets; MPGN, membranoproliferative glomerulonephritis; SLE, systemic lupus erythematosus.

In recent years, the knowledge of the pathogenesis of the hemolytic uremic syndrome has extended tremendously as shown in Figure 1.1.[4] In more than 90% of the cases, the disease is triggered by an infection with Shiga-like toxin producing *Escherichia coli* (STEC). Non-STEC-HUS is seen in 5-10% of all pediatric HUS cases, can appear at any age and may be sporadic or familial. These patients have a poor prognosis with a high mortality and morbidity in the acute phase of the disease and

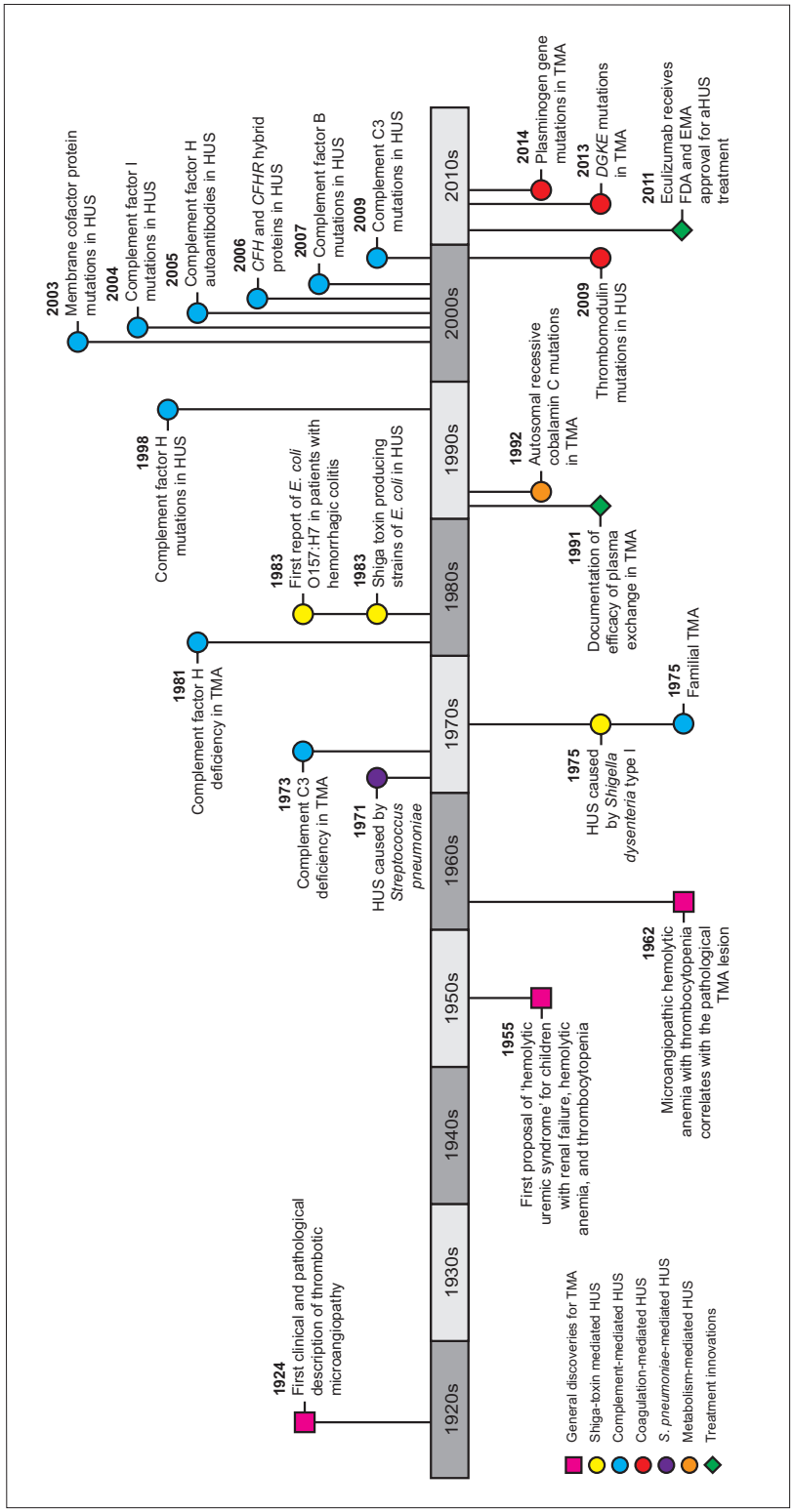

Figure 1.1. Timeline of the key events in the history of the hemolytic uremic syndrome. This figure illustrates the progression in recent years of the knowledge of the underlying causes of thrombotic microangiopathies, in particular HUS. 'CFH' stands for complement factor H, 'CFHR' complement factor H related, 'DGKE' diacylglycerol kinase ε, 'EMA' European Medicines Agency, 'FDA' Food and Drug Administration, 'HUS' hemolytic uremic syndrome, and 'TMA' thrombotic microangiopathy. *Adapted with permission from George and Nester (N Eng J Med 2014;317:654-666), Copyright Massachusetts Medical Society.*

progression to end stage renal disease (ESRD) in 50% of the cases.[5, 6] Causes of this so-called atypical HUS have been identified, as shown in Table 1.1, including disorders of complement regulation (aHUS) and various non-enteric infections such as *Streptococcus pneumoniae* infections (SP-HUS). Viruses, malignancies, drugs, bone marrow and kidney transplantation, pregnancy, and systemic diseases are associated with the disease as well.

1.1. STEC infection and the hemolytic uremic syndrome

1.1.1. Current opinions on the pathophysiology of STEC-HUS

The most common cause of the hemolytic uremic syndrome in childhood is an infection with Shiga-like toxin producing bacteria. Usually this is STEC, but *Shigella dysenteriae type 1* has been reported to cause a number of cases of HUS as well.[7,8] The reservoir of STEC is mainly the gut of cattle, in which bacteria are harmless. Fecal contamination of meat in slaughterhouses (especially ground beef), biological food product (e.g. dairy products, fruit, and raw vegetables), and water can cause the bacteria to reach the human food chain. Person-to-person transmission have been described as well.[9, 10] After oral ingestion of contaminated food and surviving the acidity of the stomach, STEC reaches the gut, where it adheres to the enterocytes of the gastrointestinal mucosa.[9] This adherence will directly lead to upregulation of the production of pro-inflammatory chemokines by the intestinal epithelial cells, resulting in an inflammation of the intestine and (bloody) diarrhea.[11] By adhering to enterocytes, STEC remains in the gut lumen and utilizes a type III secretion system (TTSS), a multi-component organelle made up of about twenty gene products.[12, 13] The TTSS is used to inject multiple virulence effector proteins directly into the infected cell to ensure that infected cell remain alive.[13] Shiga-like toxin (Stx) producing bacteria make various exotoxins, mainly Stx-1 and Stx-2. Once Stx reaches the circulation, it has to be transported to the renal endothelium to cause TMA in the kidney; Stx, however, has only once been detected in blood of STEC-HUS patients and only in the first hours of presentation.[14] Many different cell types have been suggested: erythrocytes, platelets, polymorphonuclear leukocytes, and peripheral blood monocytes have been mentioned as toxin carrier, but the exact carrier has not been identified yet.[15-20] Stx1 can induce the synthesis of several cytokines in monocytes and these locally produced cytokines may contribute in the pathogenesis of HUS.[19]

It is assumed that in the kidney, the toxin is internalized into glomerular endothelial cells by binding to the globotriaoceramide receptor (Gb_3 or CD77).[21] The presence of the inflammatory mediators TNFα, IL-1, or LPS increases the availability of Gb_3 on endothelial cells, making them more sensitive

for Stx.[22] Once bound to Gb_3, the toxin is internalized via the Golgi apparatus to the endoplasmatic reticulum (ER), a process that is called retrograde transport.[23] Stx is able to escape lysosomal degradation and becomes toxic to the ribosome after reaching the cytosol. Cleavage of 28S rRNA by the A-subunit of the toxin will inhibit protein synthesis, as protein elongation cannot occur. Furthermore, in the ER a stress response is induced that signals stress-activated protein kinase and apoptosis initiation.[23, 24] Eventually, all this can contribute to glomerular endothelial cell damage, increased thrombogenecity of the vasculature, platelet activation, and local intravascular thrombosis, with STEC-HUS as result.

1.1.2. Epidemiology and clinical course of STEC-HUS

Although over 200 different serotypes of STEC have been associated with human disease [25], most cases of HUS are associated with STEC serotype O157:H7. Other serotypes like O157:H$^-$, O26, O111, O103, and O145 have increasingly been mentioned.[26-28] The highest incidence of STEC-HUS is seen in Argentina (13.9/100,000 children under 5 years of age), which is much higher than in the U.S. where the incidence is expected to be 2.1/100,000 in general and 6.1/100,000 in children under 5 years of age.[14, 29, 30] The disease may be sporadic or present in an outbreak and more cases are seen in warmer seasons: about 60% occurs between June and September.[30]

In a five year time period (2008-2012), 2964 STEC infections were reported to the Dutch National Institute for Public Health and the Environment (RIVM); 1711 isolates were sent in for serotyping.[10] The RIVM confirmed 869 of these isolates to be indeed STEC, of which 35.6% were typed as O157 and 64.4% as non-O157. A voluntary questionnaire, including demographics, signs and symptoms, antibiotic use, and evolution of disease, was completed by 280 STEC patients. HUS was diagnosed in 13 STEC O157 patients and in four STEC non-O157 patients, with a median age of 3 years (range 0-60 years) and 29 years (range 2-81 years), respectively. Non-O157-HUS serogroups were O26 (n=2), O83 (n=1), and O non-typable (n=1).[10]

Only 5% - 15% of the people that are infected with the bacteria develop HUS. In general, young children are sensitive for the development of HUS. In the recent German STEC outbreak in 2011, however, the world's largest so far, mostly adults above 20 years and predominantly females were affected. This was attributed to the changes in the microbial characteristics of the bacteria (STEC O104:H4), which shares virulence characteristics of both typical STEC strains and enteroaggregative *E. coli* strains, indicating that changes in the bacterial characteristics can lead to changes in host profile.[31] Although a greater proportion of patients infected with STEC O104:H4 eventually developed HUS, both the clinical course of individual patients and the mortality (~4%) seemed to be

comparable to historic reports.[31, 32] Three to eight days after contamination with the bacteria, the patient evolves abdominal pains with watery and/or bloody diarrhea, followed within 24 hours by hemolytic anemia, thrombocytopenia, and acute renal failure. Anyhow, it needs to be emphasized that absence of diarrhea does not exclude the possibility of an STEC infection: in 6% of the STEC-HUS patients, there is no predomal phase of diarrhea.[33, 34] Acute renal failure is usually started with signs of glomerulonephritis (hematuria, proteinuria) and a sudden rice in serum creatinine, probably caused by an impaired perfusion due to thrombosis in the renal arterioles and glomerular capillaries with a decrease in glomerular filtration rate as result.[35]

As said before, STEC-HUS is a severe disease: seventy percent of the patients require red blood cell and platelet transfusions, 50% - 65% need temporarily dialysis, and about 25% have neurological complications at presentation an/or during the disease course.[36] Other extrarenal complications, such as pancreatic complications (~3%), have been associated with STEC-HUS as well. The mortality in children with STEC-HUS is 1% - 4% during the acute phase of the disease; about 70% of the patients completely recover after an episode of STEC-HUS.[37-39] However, it still has to be seen what the long term follow up of STEC-HUS patients will show. In a cohort of 619 STEC-HUS patients, renal survival 15 years after the onset of disease was 88%. It has been demonstrated that in case of renal damage development after the acute phase, end stage renal disease occurs more than ten years after the onset of disease.[33]

1.1.3. Diagnosis of STEC infection in HUS patients

An accurate and timely diagnosis of STEC infection in HUS patients provides the potential benefit of making the right diagnosis and guiding the clinician's care of these children. Ideally it should also include the identification of STEC O157:H7 and non-O157:H7 isolates for purposes of infection control and public health disease surveillance. Recommended for diagnosis of STEC is both a culture for specific detection of serotype O157 (enrichments steps, use of sorbitol-MacConkey agar plates) and an assay for detecting Shiga toxins (immunoassay for Stx1/Stx2, PCR for Stx1/Stx2). One should be aware that a low inoculum of STEC can be enough to develop HUS. It may therefore be advisable to examine up to three feces samples or a rectal swab in case of no stool to use. In addition to stool examination, serological investigation for the presence of immunoglobulins to the O157 lipopolysaccharide (LPS) or to LPS of several other STEC serogroups clearly adds to making the diagnosis of a STEC infection.[34, 40] An overview of diagnostic tools to be performed in patients suspected for HUS in shown in Table 1.2.

Table 1.2. Overview of investigations to be performed in patients suspected with HUS.

Underlying cause of TMA	Technique
Disorders of complement regulation	
C3 and C4 levels	nephelometry (serum)*
C3d levels	immuno electrophoresis (EDTA plasma) *,†
FH and FI levels	ELISA (serum)*
Autoantibodies against FH	ELISA (serum)*
Surface expression CD46	FACS (EDTA blood)
Mutational screening *CFH*, *CFI*, *CD46*, *C3*, *CFB*, *THBD*, *DGKE*, and *PLG*	sequencing analysis (EDTA blood)
ADAMTS13 deficiency	
ADAMTS13 activity	FRETS vWF73 (citrate plasma)*
Rare HUS causes	
Defective cobalamin metabolism	
- homocystein levels	HLPC (potassium-EDTA plasma)*,†
- methylmalonic acid levels	LC-Tandem MS (potassium-EDTA plasma)*,†
- mutational screening *MMACHC*	sequencing analysis (EDTA blood)
HIV	serology
Pregnancy	pregnancy test
HELLP syndrome	liver enzymes
Antiphospholipid syndrome	anti-phospolipid antibody
Systemic lupus erythematosus	- antinuclear antibody
	- lupus anticoagulant
STEC infection	Fecal culture and/or rectal swab, stx1 and stx2 detection by PCR/EIA, anti-O157 LPS antibody (ELISA)
Streptococcus pneumoniae infection	culture, PCR, Coombs test, peanut lectin activity test, transferrin isoelectric focusing
HUS due to medication	see Table 1.1

* Serum and EDTA plasma samples need to be centrifuged as soon as possible after sampling (preferably within 60 minutes).

† For the analysis of C3d, homocystein, and methylmalonic acid levels, (potassium-) EDTA blood needs to be placed on ice immediately after sampling.

Abbreviations: ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; CD46, membrane cofactor protein; DGKE, diacylglycerol kinase ε; FB, complement factor B; FH, complement factor H; FI, complement factor I; EDTA, ethylenediaminetetraacetic acid; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescent-activated cell sorting; FRETS vWF73, fluorescence- quenching substrate for ADAMTS13; HELLP, haemolysis, elevated liver enzymes and low platelets; HIV, human immunodeficiency virus; HPLC, high-performance liquid chromatography; LC-Tandem MS, liquid chromatography–tandem mass spectrometry; MMACHC, methylmalonic aciduria and homocystinuria type C protein; THBD, thrombomodulin.

1.1.4. Treatment options in STEC-HUS

Treatment of STEC-HUS is still purely symptomatic and is aimed to prevent gastrointestinal, hematological, vascular, and renal complications. Within the first four days of diarrhea and no signs of renal failure, volume expansion can prevent oligoanuric renal failure and STEC-HUS.[41] Many patients receive packed red blood cells to correct the low hemoglobin level. Dialysis is indicated in case of severe electrolyte imbalance, uremic symptoms, or fluid overload that fails to respond to conservative methods.[33] Antibiotic treatment in children with an STEC infection is contraindicated: it increases the risk of HUS development (RR 14.3, 95% CI 2.9-70.7), probably due to increased toxin release by the bacteria and the killing of sensitive but harmless bacteria in the gut, so that the virulent STEC can get the upper hand.[42]

Many treatments have been tested in clinically controlled trials (Stx-binding agents, intravenous IgG, plasma therapy, fibrinolytic agents, corticosteroids, antiplatelet drugs, and antioxidants), but none of them has shown to have any effect over placebo in the acute phase of the disease.[36]. Length of anuria (more than 10 days) and prolonged dialysis are the most important risk factors for a poor outcome.[39] For a good long-term renal outcome, careful blood pressure control and renin-angiotensin system blockade may be beneficial.[33, 43] For the minority who progress into ESRD (~3%)[39], kidney transplantation is the final option. The outcome of kidney transplantation in children with STEC-HUS is good, with recurrence rates of almost 0% and a graft survival at 10 years that is better than in control children whom needed a kidney transplantation due to another disease.[44]

The use of eculizumab, a complement inhibitor which is approved for atypical HUS, in the treatment of STEC-HUS is debated. In most published cases, eculizumab has been administered to most severely ill patients in which HUS was already resolving.[45, 46] Larger cohorts in the German STEC O104 outbreak showed no significant differences on mortality or on signs and symptoms of severe TMA and no controlled trial has been conducted so far.[47]

1.2. *Streptococcus pneumoniae* and the hemolytic uremic syndrome

1.2.1. Current opinions on the pathophysiology of SP-HUS

Streptococcus pneumoniae is a Gram-positive, lancet-shaped cocci and a normal inhabitant of the human upper respiratory tract. It generally causes no illness, but still is the most common cause of pneumoniae, paranasal sinusitis, otitis media, and bacterial meningitis. In 1971, a *Streptococcus pneumoniae* infection was associated with HUS for the first time (Figure 1.1).[48]

The current hypothesis of the pathogenesis of SP-HUS is the desialysation of glycoproteins of red blood cells, thrombocytes, and endothelial cells by the bacteria's neuraminidase, leading to the exposure of the Thomson Friedenreich cryptantigen (T-antigen) on the surface structures of these cells.[48, 49] Naturally occurring circulating IgM antibodies against this T-antigen can bind to the exposed T-antigen, resulting in the agglutination of red blood cells *in vitro*.[50] However, the exact role of these anti-T-antigen antibodies in the pathogenesis of SP-HUS is not known: T-antigen activation can be caused by more than 77 well-defined serotypes, most of them not associated with pneumococcal HUS.[51]

1.2.2. Epidemiology and clinical course of SP-HUS

SP-HUS is a rare complication that occurs in about 0.4-0.6% of the patients diagnosed with invasive pneumococcal disease (IPD); the incidence is highest in children under 2 years.[52, 53] As in other countries, the incidence of IPD had tremendously declined in The Netherlands since the introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) into the Dutch vaccination program in 2006: the overall incidence in children <5 years of age decreased from 43.2/100,000 to 18.4/100,000.[54] Since the introduction of PVC7, an increase has been seen in cases associated with nonvaccine-covered serotypes: for instance, the incidence of serotype 19A infection increased from 1% in the prevaccine era to 20% in the postvaccine era.[55] This serotype is not covered in the Dutch vaccination program by both PCV7 and the nowadays used 10-valent pneumococcal conjugate vaccine Synflorix. After the introduction of the pneumococcal vaccination in the US, where serotype 19A is also not included in the vaccination program, most SP-HUS cases are now associated with this serotype.[56]

SP-HUS usually develops 7 to 9 days after the onset of the symptoms related to the infection; cases are associated with pneumonia, empyema, and meningitis.[53, 57] The high incidence of empyema (an accumulation of pus in a natural existing anatomical cavity), which is found in 51-66% of SP-HUS cases associated with pneumonia, suggests that a heavy bacterial load may increase the risk of HUS in the setting of pulmonary infection.[50, 58]

Patients with SP-HUS are usually younger at presentation and have a more severe disease course than patients with STEC-HUS. A higher morbidity (25-50%) and mortality (2-12%) rate has been reported over the years as compared to STEC-HUS.[50, 57, 58] Patients have more frequent need for acute dialysis, require more platelet and red blood cell transfusions, and have a much longer duration of thrombocytopenia.[50] These are two-to-three times higher rates than in patients with STEC-HUS. The mortality rate is highest in patients with pneumococcal meningitis complicated by HUS: in literature reports, 28% of the patients with SP-HUS have meningitis, but 88% of the deaths

occurred in this group.[53] The poor prognosis in patients with pneumococcal meningitis is thought to be coherent with the observation that in severe meningitis the neuraminidase activity in the central nervous system is elevated.[59]

Because of limited studies and short follow-up periods, the long-term outcome of SP-HUS is not clear yet. End-stage renal disease occurs in 10-16% of the patients.[57] Percentages of patients that remain with sequelae like elevated serum creatinine, proteinuria, hypertension, or neurological sequelae range from 16-60%.[53, 57] It seems that the greatest risk for the development of long-term renal sequelae is the need for dialysis for more than 20 days.[56]

1.2.3. Diagnosis of SP-HUS

Diagnosis of SP-HUS can be challenging due to the clinical similarity to disseminated intravascular coagulation. The lack of familiarity with SP-HUS by the general pediatrician and other pediatric specialists can delay the diagnosis: in one case series, almost every diagnosis was made by a nephrologist, and for half of the patients the diagnosis was overlooked by other specialists.[56]

Clinical symptoms and laboratory findings involve evidence of hemolytic anemia, thrombocytopenia, acute renal failure, a positive result of a peanut lectin activity test, a positive result of a Coombs test, a normal of slightly elevated prothrombin time, and a abnormal transferrin isoelectric focussing (IEF) pattern (Table 1.2).[53] Evidence of *Streptococcus pneumoniae* in blood or cerebrospinal fluid might be absent, as only 10%-30% of the patients with pneumonia have a positive blood culture.[56]

The sensitivity of the peanut lectin activity test, which measures the exposure of T-antigen, is very high (100%), but unfortunately, the specificity is only 48% for SP-HUS: patients with uncomplicated invasive pneumococcal disease and pneumococcus-associated hemolytic anemia will have a positive result as well.[60] The Coombs test, which detects the T-antigen-antibody interaction at the plasma membrane of erythrocytes, is highly specific for the disease (90%) [60], but it is not known what the incidence is in other forms of pneumococcal infection. The transferrin IEF in SP-HUS patients differs from patterns in other hypoglycosylation diseases, like CDG I and alcohol abuse, and from patterns in other forms of HUS, which means that it is possible to dissociate SP-HUS from these clinical entities on the basis of transferring IEF.[61]

1.2.4. Treatment options in SP-HUS

In SP-HUS, it is essential to control the abnormalities caused by the renal failure, hemolytic anemia, and thrombocytopenia by means of transfusion of red blood cells and/or platelets. Dialysis or hemodiafiltration is often required to restore fluid and mineral abnormality and to treat the renal

failure. Transfusion of washed red blood cells or platelets is preferred, due to the presence of anti-Thomsom Friedenreich IgM antibodies in unwashed blood products. For the same reason it is important to avoid priming the dialysis system with unwashed blood products prior to hemodiafiltration in young children.[53] The use of fresh-frozen plasma or plasma products is associated with increased hemolysis and needs to be avoided.[62] To treat the pneumococcal infection, antibiotics like vancomycin and an extended-spectrum cephalosporin are needed.[63] In literature, no evidence is found that plasmapheresis is of any value in the treatment of SP-HUS.

1.3. The complement system and the hemolytic uremic syndrome

1.3.1. Current opinions on the pathophysiology of aHUS

Already in the seventies of 20th century decreased plasma levels of the complement proteins C3 and complement factor B (FB; encoded by the gene *CFB*) in both sporadic and familial cases of HUS have been identified.[64] The presence of increased breakdown products of these proteins suggested an activation of the alternative pathway of the complement system could be involved in the pathogenesis of the disease.[65] In the last decade, evidence has been shown between these so-called atypical HUS (aHUS) and genetic abnormalities in complement (regulating) genes, resulting in hyperactivation of the complement system, eventually leading to glomerular endothelial activation and thrombosis.

Activation and regulation of the complement system

The human complement system is part of the innate immunity and consists of more than 40 plasma and membrane-associated proteins. The most important roles of the complement system are the recognition of pathogens (opsonization), the activation and chemotaxis of leukocytes, and the induction of cell lysis by incorporation of the membrane attack complex (MAC).[66, 67] Three activation pathways are recognized: the classical pathway, the mannose binding lectin pathway, and the alternative pathway. The latter is mostly affected in aHUS.

To prevent continued and unopposed complement activation on host cells and resulting cell damage, the complement system is tightly regulated. Each pathway has its own regulators (inhibitors), but some regulators work on more than one pathway (Figure 1.2; inhibitors shown in italic). Non-host surfaces that either lack membrane-bound regulators, do not produce their own regulators, or cannot bind soluble regulators are attacked and damaged by the complement system.

The key regulators of the alternative pathway are complement factor H (protein: FH; gene: *CFH*), complement factor I (protein: FI; gene: *CFI*), and membrane co-factor protein (protein: MCP or CD46; gene: *CD46*). These complement regulatory proteins are either constitutively present on the endothelial cell membrane or are bound by the endothelial glycocalyx. The normal mechanism of complement regulation at the cell surface by these regulators is schematically shown in Figure 1.3A.

Mutations in complement genes in aHUS patients

A loss-of-function mutation in a complement regulating gene, or a gain-of-function mutation in a gene that encodes a complement activator, will lead to an uncontrolled activation of the complement system, resulting in formation of the membrane attack complex on cell surfaces of especially endothelial cells in the microcirculation of the kidney. As a result, endothelial cells are activated and damaged and leukocytes are attracted, releasing oxygen radicals and proteinases, which can further damage the endothelium. This eventually will result in increased platelet adherence and the formation of microthrombi in the kidney, thus explaining the characteristics triad of aHUS: acute renal failure, thrombocytopenia, and hemolytic anemia.

In 1998, Warwicker *et al*. were the first to describe a mutation in the gene encoding FH in familial cases of aHUS.[68] Nowadays, a genetic aberration in one of the proteins of the alternative complement pathway can be found in at least 50% of the aHUS patients. Mutations, usually heterozygous, have been identified in the complement inhibitors FH, FI, CD46, and FHR-5, and in the activators C3 and FB (Figure 1.1).[69-77] Mechanisms of disease for several mutations are shown in Figure 1.3. In several patients, mutations have been identified in more than one complement gene.[72, 78] In addition, aHUS is associated with the presence of a combination of single nucleotide polymorphisms in FH (FH_{TGTGT} haplotype) or CD46 ($CD46_{GGAAC}$ haplotype).[79, 80] Not unexpected, aHUS can also be caused by antibodies that impair the action of the complement regulatory proteins. Thus far, autoantibodies against FH (αFH) have been identified in aHUS patients.[81] These αFH autoantibodies can block the epitopes of FH that are involved in binding to the endothelial cell membrane, resulting in defective regulation of the complement at the site of the endothelium, which can lead to endothelial damage.[82] The development of these αFH antibodies is associated with a polymorphic homozygous deletion of complement factor H related proteins (FHR-1 and FHR-3; encoded by *CFHR1* and *CFHR3*).[81] In only a few studies, mutation have been identified in genes that are not solely involved in the complement system, but also in the coagulation system (Figure 1.1). Thrombomodulin (*THBD*) was the first coagulation gene in which pathogenic mutations were

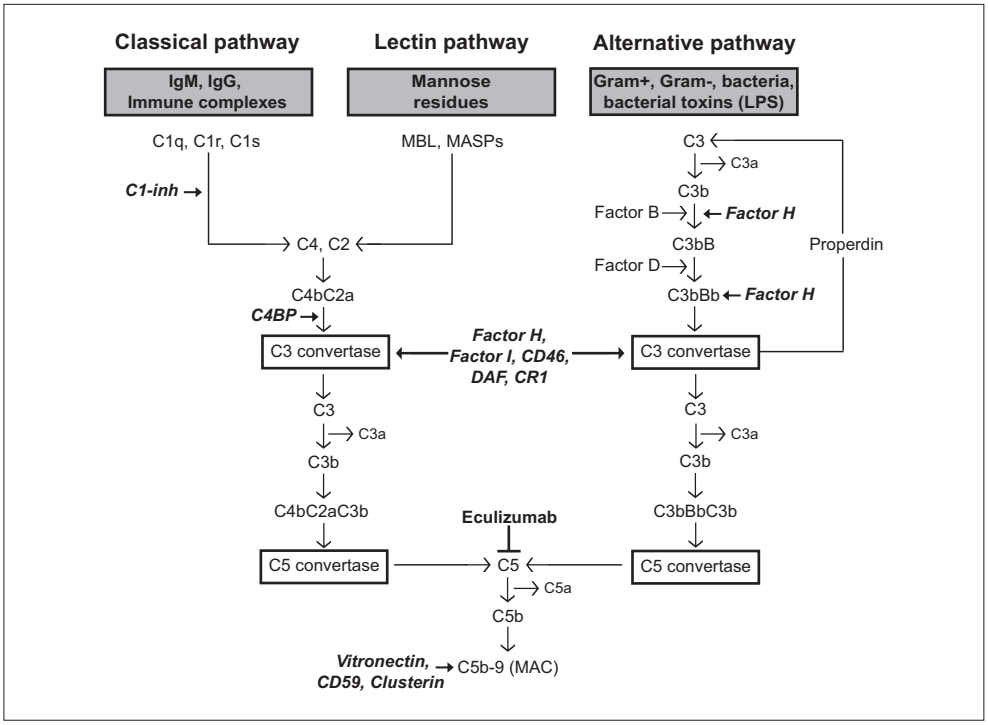

Figure 1.2. Schematic overview of the three activation pathways of the complement system. Activation via the classical, lectin, or alternative pathway leads to production of C3 convertases, that can cleave C3 in C3b and the anaphylatoxin C3a. Active C3b finally initiates the production of membrane attack complexes that can cause lysis of the cells. The regulators of the complement system, important in the protection of host cells against complement activation, are shown in italic. Abbreviations: C1-inh, C1 inhibitor; C4bp, C4 binding protein; CR1, complement receptor-1; DAF, decay accelerating factor; MAC, membrane attack complex; MASP, mannose associated serine protease; MBL, mannose binding lectin; CD46, membrane cofactor protein.

described.[73] Thrombomodulin still has a link with the complement system, as it can regulate the alternative pathway via the inactivation of C3b by Factor I.[73] The second coagulation gene associated with aHUS does not have a clear link with the complement system. Loss of function of diacylglycerol kinase ε (encoded by *DGKE*) might result in a prothrombotic state, but the exact mechanism how this can result in aHUS is not yet clear.[83] At first, *DGKE* mutations were associated with normal complement levels, but recently a homozygous nonsense mutation in *DGKE* was identified in a family with hypocomplementemic aHUS; no other cause of these low complement levels could be found in associated complement genes or in the presences of αFH.[84] Very recently, mutations were identified in a third coagulation protein, plasminogen (*PLG*), the precursor of plasmin that can degrade fibrin clots. In 4 out of 36 aHUS in which next generation sequencing (targeted genomic enrichment and massively parallel sequencing) was performed, a potentially pathogenic variation was identified in *PLG*.[85] It needs to be mentioned that three of these patients had deleterious

nonsynonymous rare variants in complement genes as well. In this specific study, *PLG* had the second greatest number of mutations after *CFH*, but this high mutation rate still needs to be confirmed in other patient cohorts. Recently, in five patients with the rare and severe combination of pulmonary arterial hypertension and renal TMA, an underlying genetic defect was shown in *MMACHC*, resulting in a cobalamin C deficiency. Only two patients developed full-blown hemolytic uremic syndrome and just these patients had a defect in the complement system as well (*CD46* mutation and αFH).[86] Several case reports of STEC-HUS and SP-HUS patients with complement mutations have been published, but large cohorts still need to be investigated.[87-91]

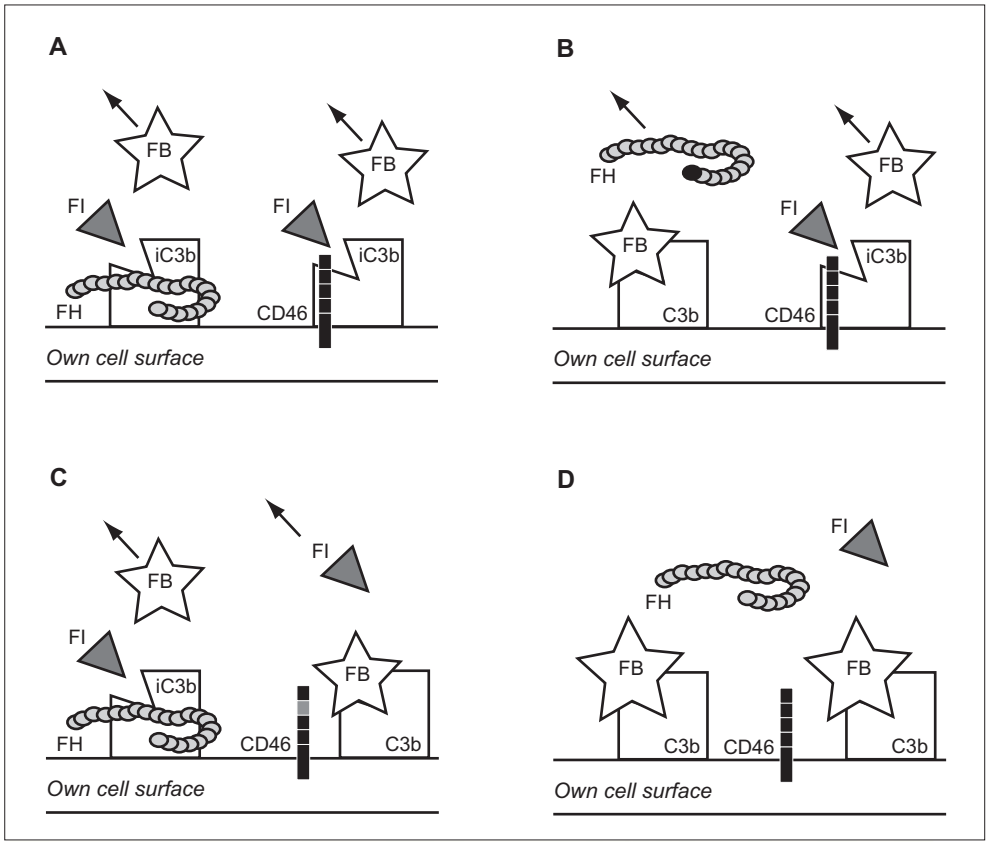

Figure 1.3. Schematic model for the mechanism of alternative complement pathway regulation at host cell surfaces. In normal circumstances small amounts of C3b are deposited on cell surfaces, but these molecules are rapidly eliminated by FI, with the help of the cofactors FH and CD46 (**A**). In case of a loss-of-function mutation in FH (**B**) or CD46 (**C**), deposited C3b on host cells cannot be efficiently be eliminated. This is followed by complement activation that can lead to complement mediated damage of plasma exposed cells, such as glomerular endothelial cells. In case of a gain-of-function mutation in a molecule that participates in activation of the alternative pathway (C3, FB), endothelial cells will be damaged despite the presence of functional regulators (**D**). *Adapted with permission from Jokiranta et al (Mol Immunol. 2007;44:3889-3900.), Copyright Elsevier Limited.*

Incomplete penetrance of aHUS

Mutations in complement (regulating) genes can be found in healthy family members: the penetrance of disease among carriers of mutations in *CFH*, *CFI*, and *CD46* is approximately 50-60%.[29, 71, 92] This indicates that the genetic aberrations are probably important for the development of aHUS, but not the sole cause. Affected patients may carry combined mutations, in more than one gene [71, 72], or carry a mutation in combination with the associated FH or CD46 haplotype (complotype).[92] Family members whom only carried one mutation or no polymorphisms were not affected [79], but this could be due to incomplete penetrance as well. Atypical HUS may not occur until adulthood, even in patients with multiple genetic defects. This indicates that an environmental factor, like a complement trigger, is probably needed to develop the disease. For instance, Caprioli *et al*. reported that in 77% of the patients with a mutation in *FH*, *FI*, or *CD46*, the clinical symptoms were preceded by flulike symptoms, gastroenteritis, or other infections[71]. This was also reported by Geerdink *et al*.[93]

1.3.2. Epidemiology and clinical features of aHUS

The incidence of aHUS is not known precisely, but is estimated to be ~2 per million in the United States.[78] Instead of STEC-HUS, in which in general more pediatric patients are seen, aHUS can arise at any age (1 day – 83 years). Patients with *DGKE* mutations are usually diagnosed before 1 year of age and autoantibodies against Factor H are seen mostly in children between 7 and 11 years[78]; in our cohort, all patients with *C3* mutation presented with aHUS in adulthood.[94] Sporadic and familial cases have been identified. In more than half of the cases, the disease is triggered by an infectious event, mainly upper respiratory tract infection or diarrhea/gastroenteritis, and fever, making the differentiation between STEC-HUS and aHUS sometimes difficult.[29, 71, 93] Extrarenal manifestations are reported in only 10% to 20% of the patients, mostly neurological[95]; as in other chronic kidney diseases, vascular complications are seen in patients on dialysis (unpublished data Loirat *et al.*).

1.3.3. Diagnosis of aHUS

As many clinical symptoms in aHUS overlap those in STEC-HUS, the diagnosis of aHUS is difficult. An overview of investigations to be performed in patients with HUS is shown in Table 1.2. STEC infection has to be ruled out in pediatric patients and considered in adults, as unusual presentation of STEC-HUS can occur, i.e. without diarrhea (~10) or at older age as in the German outbreak.[35] The presence of ADAMTS13 deficiency needs be investigated to exclude TTP: an activity below 5-10% could indicate acquired anti-ADAMTS13 autoantibodies (in the majority of the patients) or a genetic

abnormality. Other rare HUS causes, like a defective cobalamin C metabolism, *Streptococcus pneumoniae* infection, and the HELLP syndrome should be considered and investigated at presentation.

Recent guidelines recommend the screening for complement abnormalities in patients with aHUS.[96, 97] Complement activity (CH50 and AP50) and serological complement components (C3, C4, C3d, FH, and FI) can be measured in serum or plasma, drawn before the start of therapy. It must be realized that most assays measure the presence and not the activity of the protein. Moreover, abnormalities in complement regulation may only occur at the level of the endothelial cell surface, and not systemically.[98] Therefore, serological levels of individual proteins may be normal in patients with complement dysregulation and thus cannot not exclude a genetic complement disorder.[5, 71, 99] The surface expression of membrane-bound CD46 on mononuclear leukocytes can be investigated by fluorescent-activated cell sorting (FACS), but again: normal expression does not rule out impaired function of CD46.[100] Mutational screening should be performed in the complement and coagulation genes that have been associated with aHUS (*CFH*, *CFI*, *CD46*, *C3, CFB, CFHR5, THBD, DGKE, PLG*), irrespective of serum C3, FH, or FI levels. The gene encoding cobalamin C (*MMACHC*) can be screened for abnormalities once a cobalamin C deficiency is detected. The presence of αFH can be identified in serum by enzyme-linked immunosorbent assay. An overview of the investigations to be performed in patients with aHUS is shown in Table 1.2.

1.3.4. Treatment options in aHUS

Treatment must be started urgently to be effective, preferably within 24 hours after diagnosis. At this moment it takes up to one to three months to perform genetic studies that are needed to specify the underlying cause. Furthermore, currently still no complement abnormalities can be found in the 40% of patients. Therefore, it is advised to start plasmapheresis, which replaces missing or deficient proteins and removes disease causing antibodies, as the first treatment option when the diagnosis is still not clear. New treatment options such as complement inhibitors are now available as first line treatment in aHUS as well, especially for those patients that do not respond to plasmapheresis in the first days.

Plasma therapy

In the most recent guidelines, it is recommended to start plasmapheresis within 24 hours of diagnosis.[96, 97] It is suggested to exchange 1.5 times the expected plasma volume (60-75 ml/kg) and replace plasma with fresh frozen plasma or virus-inactivated pooled plasma. There are no evidence

based treatment schedules for plasmapheresis treatment reported in literature. As CD46 is a membrane-bound protein and a defect CD46 protein can therefore not be substituted by plasma therapy, this has limited value in the treatment of aHUS. Remission is achieved anyhow in 80-90% of patients with *CD46* mutations without plasma treatment.[29]. However, since it is not known which complement genes are involved in the pathogenesis of aHUS at first presentation and since it is known that combined mutations in complement genes can occur, plasma therapy still has its place in treatment of aHUS. Besides plasmapheresis, avoiding triggers of endothelial injury, such as hypertension and hypercholesteraemia, by adequate blood pressure control and the use of statins are important treatment options in the acute phase of the disease and should be maintained once in remission.

Complement inhibiting therapy: eculizumab

In 2012, the recombinant, humanized, monoclonal anti-C5 antibody eculizumab (Soliris®, Alexion Pharmaceuticals, Cheshire, CT, USA) has been registered for the treatment of aHUS patients by the FDA and EMA. Eculizumab specifically binds to C5, thereby blocking the cleavage of C5 into the anaphylatoxin C5a and C5b and the formation of the membrane attack complex C5b-9 is prevented *in vitro* (Figure 1.1). In one patient with the acquired pregnancy condition of hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome, however, C5a levels increased despite eculizumab treatment, while C5b-9 generation was blocked.[101] This indicates that the enzymatic generation of C5a *in vivo* might not be blocked by eculizumab, but further research is needed.

Eculizumab has been approved worldwide for the treatment of paroxysmal nocturnal hemoglobinuria (PNH), a hematological disease associated with loss of regulation of the terminal complement pathway on erythrocytes.[102] Since the first successful reports of eculizumab treatment in aHUS patients in 2005, many reports have followed, describing patients that received eculizumab to rescue their native kidneys or to prevent a recurrence in a graft after transplantation (reviewed by Loirat *et al*.[78] and Köse *et al*.[103]).

Two international multicentre prospective phase 2 open-label clinical trials in adolescent and adult aHUS patients and a retrospective study in children have been conducted so far.[104] The results showed that thrombocyte levels increased and renal function improved already from the first dose of eculizumab. None of the patients required a TMA intervention (plasmapheresis or dialysis) during the treatment. Eculizumab was well tolerated in these clinical studies. Adverse effects that were most frequently reported were hypertension, upper respiratory tract infection, and diarrhea. Treatment with eculizumab does increase the risk of severe infection and sepsis. As immunity

against *Neisseria meningitis* depends on the terminal complement pathway, prevention of meningococcal infection by vaccination or antibiotic prophylaxis is crucial in patients treated with eculizumab. Children need to be vaccinated against pneumococcus and *Haemophilus influenza* as well.

Eculizumab is very expensive: current estimates are up to 500,000 Euro's per treatment year. The burden of two-weekly visits to the hospital for the treatment is high. Although this drug certainly has changed the future perspectives of patients with aHUS, many unsolved questions remain: who should receive the drug, which optimal treatment schedules should be used, and how long should therapy be continued. It is even undecided if prophylactic treatment is needed. Cost effectiveness should be evaluated in carefully conducted prospective cohort studies.

Transplantation

The clinical outcome of renal transplantation in patients with aHUS is dismal. Approximately 50% of patients with aHUS will develop recurrent disease and graft loss. There are no clinical predictors for the outcome of transplantation, although the use of a calcineurin inhibitor after transplantation is associated with an even higher recurrence rate.[105] Unfortunately, patients with aHUS are also more prone to develop acute rejections, which also affects graft survival. Knowledge of the underlying genetic defect is helpful in predicting prognosis. The recurrence risk in patients with a *FH* mutation is 75-90%, for patients with a *FI* mutation this is 45-80%, and in case of a *C3* mutation, the risk of an aHUS recurrence is 40-70%.[106] Recurrences have been seen in patients with *FB* and thrombomodulin mutations, as well. On the other hand, patients with a mutation in the gene encoding the membrane-bound CD46 have a low risk to develop a disease recurrence in the graft.

Knowledge of the underlying genetic defect is also critical when considering a living-related kidney transplantation. Until recently, living kidney donations were considered unjustified in patients with aHUS: there is not only a high risk of graft failure in the recipient, more importantly the donor may be a carrier of the mutation and could develop aHUS due to uncontrolled complement activation during the donor procedure.[107] If a mutation is identified in the acceptor, family members can be screened for this mutation, and only donors without this mutation can be accepted for donation. Of note, if no mutations are found, current policy is to not accept any related donor, as genetic aberrations may be present in not yet associated genes.

The burden of endothelial injury in a post-transplantation setting, caused for instance by immunosuppresive drugs, viral infections or rejection, might trigger *de novo* HUS in the presence of mild genetic susceptibility to HUS.[108] Possible causes of recurrent and *de novo* post-transplant HUS,

both genetic and environmental, are shown in Figure 1.4. Although the influence of environmental factors leading to endothelial injury is probably higher in *de novo* HUS, genetic aberrations in the complement system are still found in 30% of the patients diagnosed with *de novo* post-transplant HUS.[108] To minimize the environmental risks, adequate control of blood pressure and hypercholesterolemia in combination with the prudent use of calcineurin inhibitors during renal transplantation is warranted.[108]

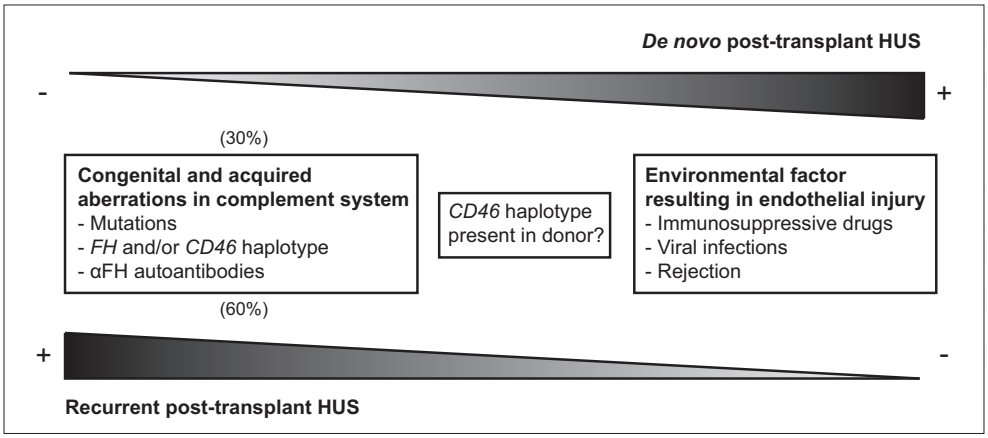

Figure 1.4. Genetic and environmental factors associated with post-transplant HUS. Endothelial injury in the graft can occur due to genetic aberrations in the alternative pathway of complement system (more often in recurrent post-transplant aHUS) or by environmental factors (more often in *de novo* post-transplant HUS). The presence of the $CD46_{GGAAC}$ haplotype, associated with the pathogenesis of aHUS, in the donor could influence a recurrence as well, although this has not been investigated extensively.

1.3.5. Outcome in aHUS

Without treatment, the prognosis of patients with aHUS is poor, up to 25% of patients may not survive the acute phase and up to 50% of the patients progress to ESRD.[5, 6] The outcome of disease is dependent on the underlying genetic aberrations. Eighty to 90% of the patients with a mutation in the membrane-bound CD46 protein often go into remission, although recurrences often occur.[29, 71] In contrast, 60-70% of the patients with a mutation in a fluid phase protein (FH, FI or C3) will develop terminal renal failure within one year after diagnosis; in patients with αFH this amount is 30%.[109] Not many patients with an aberration in *CFB* have been reported yet, but in one study with seven aHUS patients of a *CFB* mutation, six of them lost renal function within two year after diagnosis.[74] The underlying complement defect also determines whether therapy is needed and if it will be effective. For instance, in patients with an *CD46* mutation alone, plasma therapy is of limited added value: remission is achieved in 80-90% of these patients without plasma treatment.[29] Since

CD46 is a membrane-bound protein, a defect CD46 protein can therefore not be substituted by plasma therapy.

Most recent literature data suggest that aHUS outcome may have improved over the last decade, especially in children.[109] This improvement already occurred in the era before the introduction of complement inhibition therapy and might be related to early and more intensive plasmapheresis treatment and improved conservative treatment options. The recent registration of eculizumab has dramatically improved the outcome further in aHUS patients: in the vast majority of patients in the trials, TMA activity disappeared, plasmapheresis therapy could be stopped permanently, estimated glomerular filtration rate increased, and dialysis could be discontinued.[104]

1.4. The complement system in STEC-HUS and SP-HUS

1.4.1. Complement and STEC-HUS

The first reports of involvement of the complement system in HUS in general have been published before the etiology of STEC-HUS, SP-HUS and aHUS were known. More than thirty years ago, when breakdown products of C3 and FB were found in children with HUS. At that time, the different causes of HUS were still unknown and it was not clear whether these patients had STEC-HUS or not.[65] In 1990, however, low serum C3 levels and renal C3 deposition were reported in HUS patients preceded by an STEC infection.[110] In 2009, a report was published about elevated plasma levels of complement fragments of the alternative pathway (only FB and sC5b-9 were measured) in STEC-HUS patients during the first month after onset of the disease, but a small research population was used (seventeen patients) and only four control patients that were not age-matched were included.[111] Their results were confirmed in a Swedish cohort of 10 STEC-HUS patients, in which C3a and sC5b levels were increased in the acute phase of disease and normalized in the convalescent phase.[112] Ståhl *et al*. also showed complement activation on blood cell particles in STEC-HUS patients.[112] So, complement activation has clearly been shown in patients with STEC-HUS, but it is not known whether this activation is a purely infection-related phenomenon or if it is part of the pathogenesis of the disease.

In recent years, investigators have sought for a possible active role of the complement system in STEC-HUS by shiga toxin. Orth *et al* showed the direct binding of purified Stx2 to the main complement regulator factor H.[113] The binding sites for Stx2 were mapped to short consensus repeats (SCRs) 6-8 and 18-20 of FH, both regions that are involved in surface recognition and

membrane binding of the protein.[114] Binding of Stx2 resulted in a delayed cofactor activity on the cell surface, but not in the fluid phase [113], which is also seen for loss-of-function mutations in *CFH*. Stx2 can further modulate the expression of the membrane-bound regulator CD59, but not that of CD46 and CD55, on glomerular endothelial cells, resulting in enhanced complement activation on the cell surface.[115] *In vivo*, coinfection with Stx2 and LPS resulted in glomerular endothelial C3 deposition, thrombocytopenia, and renal function impairment in a murine model of STEC-HUS. This was not observed in FB-deficient mice, indicating the importance of the alternative complement pathway. Stx1 can trigger complement C3 activation at the cell surface *in vitro*.[116] It needs to be mentioned that in all these experiments, the used Stx concentrations were much higher than the levels that were measured in STEC-HUS patients and the exact role of complement binding and activation by Stx1 and Stx2 in humans therefore remains unclear.[14]

Not only shiga toxins can manipulate the activation of the complement system. The STEC autotransporter EspP was shown to cleave C3/C3b and C5 and decrease complement activation in serum.[117] The zinc metalloprotease StcE, which is found in STEC O157:H7, is able to downregulate the classical complement pathway.[118] These examples show that STEC is very well able to manipulate complement in multiple ways.

The use of complement inhibition in STEC-HUS has been prompted by a letter to the *New England Journal of Medicine*, in which the treatment of three severely affected HUS patients with neurological involvement was described.[45] Eculizumab was given late in the disease (5-10 days after the onset) and HUS was already resolving in each patient (decreasing LDH and/or rising thrombocyte count). Two of the patients also received plasma exchange and/or dialysis prior to eculizumab initiation. Therefore, it is unclear from this case series that the improvements were due to eculizumab treatment or were a normal recovery process seen in STEC-HUS. Due to this publication and the public pressure, the manufacturer made the off-label use of eculizumab in the German O104:H4 outbreak in 2011 possible. The exact influence of complement inhibition on the outcome of these patients is not clear, but in larger cohorts, no significant differences were seen on mortality or on signs and symptoms of severe thrombotic microangiopathy complications.[47, 119, 120] In most cases, eculizumab was administered to the most severely ill patients (e.g. neurological involvement, need for renal replacement therapy) and a selection bias is present. Therefore, the need for a randomized controlled trial, in which eculizumab treatment in STEC-HUS is prospectively evaluated, is warranted.

1.4.2. Complement and SP-HUS

Animal studies have demonstrated that the complement system has an significant role in immunity to pneumococcal infection.[121-124] Furthermore, in several microbiology studies, it has been shown that *Streptococcus pneumoniae* have the capacity to bind complement factor H by their produced proteins PspC and Tuf, thereby elongating their survival in the host.[125-128] Other pneumococcal proteins, including the PspC family member Hic and the glycolytic enzyme phosphoglycerate kinase can inhibit the formation of the membrane attack complex on the surface of *S. pneumoniae*.[129, 130] Furthermore, during autolysis of the bacteria, the important virulence factor pneumolysin is released, which activates the complement system, leading to complement depletion and reduced opsonisation.[131]

1.5. Aims and outline of this thesis

Dysregulation of the alternative pathway of the complement system due to genetic mutations is believed to play an essential role in the pathogenesis of the atypical form of the hemolytic uremic syndrome. Complement factor H, the most important regulator of the alternative pathway and protector of host cells against complement activation, had been established to be the most frequent mutated protein in the genetic analysis of aHUS patients. In earlier studies, it has been shown that *Streptococcus pneumoniae* and STEC both have mechanisms to evade the complement system, thereby protecting itself against complement activation. Taken into account: 1. the importance of a dysregulation of the complement system in the atypical form of HUS, 2. the knowledge that bacteria use these complement regulators to survive in the host and 3. the similarity of the clinical manifestations of atypical and infection-related HUS, the aim of this thesis is to study and define the role of the complement system in three forms of HUS (STEC-HUS, SP-HUS, and aHUS). If the complement system has an important role in the pathogenesis of infection-related HUS as well, new therapeutic possibilities, for instance complement inhibitors, may have a place in the treatment of STEC-HUS and SP-HUS.

In **Section I** of this thesis, the mutational screening of genes encoding associated complement (regulating) proteins in a research group of 70 aHUS patients will be described. In **Chapter 2**, we examine *FH*, *FI*, *CD46*, and *FB*, and the presence of autoantibodies against FH; the results of the *FHR-5* screening are shown in **Chapter 3**. The functional consequences of newly identified *C3* mutations are investigated in **Chapter 4**. In nine aHUS patients in which no mutations had been identified

previously, we performed next generation sequencing; the results of this study are described in **Chapter 5**. The genotype – phenotype correlation in our pediatric aHUS population has been studied in **Chapter 6**. The difficulties a physician can be faced with during the therapeutic management of a patient with both STEC-HUS and hemophilia A are displayed in **Chapter 7**.

To further define the role of complement dysregulation in all forms of HUS, infection-related or atypical, we executed several biochemical studies, of which the results are depicted in **Section II** of this thesis. It is thought that the dysregulation of the complement system in aHUS occurs mostly at the level of the glomerular endothelium and not in the fluid phase[98], but on the other hand, alternative pathway activation products are present of aHUS patients. To further investigate complement activation in the circulation and to identify potential biomarkers for diagnosis, in **Chapter 8** we will evaluate the levels of complement activation in the acute phase and in remission in aHUS patients of all ages with the currently available sophisticated methods. As potential biomarkers might be able to discriminate between infection-induced HUS and aHUS at time of diagnosis, this study is extended to pediatric STEC-HUS, aHUS, and SP-HUS patients, and age-matched controls in **Chapter 9**. Although it has been shown that factor H can bind to capsulated bacteria such as *Streptococcus pneumoniae* and *Neisseria meningitidis*, no comprehensive research has been performed on the possibility of complement modulating proteins in STEC. Possible mechanisms of the STEC bacteria to evade the complement system are investigated in **Chapter 10**. To test the hypothesis that both a key environmental trigger and defective host complement regulation are required for HUS to develop, mice deficient in factor H were infected with an invasive and mutated *Streptococcus pneumoniae* species. In **Chapter 11**, the results of this study are described. Finally, in **Chapter 12**, a general discussion of the findings reported in this thesis and an outline of future perspectives are displayed.

Section I: Genetic screening in HUS

Chapter 2

Genetic disorders in complement (regulating) genes in patients with atypical hemolytic uremic syndrome

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Abstract

Atypical HUS is thought to be caused by predisposing mutations in genes encoding complement (regulating) proteins, such as Factor H (*CFH*), Factor I (*CFI*), membrane co-factor protein (*MCP* or *CD46*), and Factor B (*CFB*), or by autoantibodies against factor H (αFH) in combination with a homozygous polymorphic deletion of the genes encoding complement Factor H related proteins 1 and 3 (*ΔCFHR1/3*). The clinical impact of this knowledge is high, as it might be a prognostic factor for the outcome of renal transplantations and kidney donations. Mutational screening, by means of PCR and DNA sequencing, is performed in the above mentioned genes in a group of 72 aHUS patients. Also, the presence of αFH and *ΔCFHR1/3* was tested in patients and controls. In 23 patients, a genetic aberration in at least one gene or the presence of αFH was found. A heterozygous mutation was observed in *CFH* in nine patients, in *CFI* in seven patients, and in *CD46* in three patients. No mutations were observed in *CFB*. Seven patients presented αFH, of whom five also carried *ΔCFHR1/3* Three patients carried a combined mutation (two patients: *CFI* and *CD46*:; one patient: *CFI*, αFH, and *ΔCFHR1/ 3*). A significant difference between patients and controls was detected for the presence of all three associated polymorphisms in *CFH*. Genetic abnormalities or the presence of αFH were detected in 31.9% of the aHUS patients. Furthermore, bigenic mutations were present, indicating that routine DNA mutation analysis of all complement factors associated with aHUS is important.

Introduction

Hemolytic uremic syndrome (HUS) is a rare and severe disease, which is characterized by thrombotic microangiopathy, hemolytic anemia, thrombocytopenia and acute renal failure.[36] In most cases, HUS is seen in childhood, is preceded by watery or bloody diarrhea, and is caused by Shiga-like toxin (Stx-) producing *Escherichia coli* (STEC).[132, 133] Non-STEC-HUS is seen in 5% to 10% of all HUS cases; these patients have a much poorer prognosis. Up to 50% of these so-called atypical cases progress to end-stage renal disease (ESRD) and 25% may result in death during the acute phase of the disease.[37, 134-136] In recent years, a clear link has been established between atypical HUS (aHUS) and genetic abnormalities in regulator genes of the alternative pathway of the complement system. Mutations have been described in genes encoding complement factor H (protein: FH; gene: *CFH*), complement factor I (FI; *CFI*), and membrane co-factor protein (MCP or CD46), three important regulatory proteins of the alternative pathway.[69-71, 80, 136] Up to now, mutations in aHUS patients are predominantly found in the *CFH* gene and in lesser amounts in *CFI* and *CD46*. Very recently, the presence of autoantibodies against FH (αFH) in combination with a homozygous polymorphic deletion of complement factor H-related genes *CFHR1* and *CFHR3* (*ΔCFHR1/3*), have been associated with aHUS [81, 137], as well as mutations in genes encoding complement factor B (FB; *CFB*)[74], complement C3[75], and thrombomodulin[73].

Knowing the genetic variations present in aHUS patients is important, because it can be of prognostic value for the outcome of renal transplantation and kidney donations. In general, around 50% of the patients that underwent renal transplantation had a recurrence of the disease in the graft and graft failure occurred in more than 90% of them.[71] However, several groups have shown that patients with a mutation in the *CFH* or *CFI* gene have a worse outcome after kidney transplantation (recurrence: 80-100%) than patients with a *CD46* mutation (recurrence 0–20%).[71, 138, 139]

In the present study, mutations in genes encoding (regulating) proteins of the alternative pathway of the complement system (*CFH, CFI*, *CD46*, and *CFB)*, and the presence of αFH in combination with *ΔCFHR1/3* were studied in a cohort of 72 Dutch and Belgian patients diagnosed with aHUS. Of 19 patients, EDTA serum was available to identify the presence of αFH.

Subjects and methods

Study population

The research population consisted of 72 aHUS patients (33 children and 39 adults; age 5 months to 55 years at onset), referred to the Pediatric Nephrology Centre of the Radboud university medical centre. All patients were diagnosed with atypical, non-STEC HUS. The patients were of Dutch or Belgian origin, mostly with a Caucasian background. In ten patients from four families the familial form of aHUS was identified; the other 62 patients were diagnosed with sporadic aHUS. Permission to study DNA material was given by all patients or their parents.

Genetic analysis of genes encoding CFH, CD46, FI, and FB

Genomic DNA, isolated from peripheral blood leukocytes as described by Miller *et al*.[140], was amplified for *CFH* (NCBI RefSeq NM_000186), *CFI* (NM_000204), *CD46* (NM_172361), and *CFB* (NM_001710) by means of PCR. Primer data are available on request. Fragments included both DNA sequences of the individual exons, and the splice donor and acceptor site. The amplimers were subjected to double stranded DNA sequence analysis on an ABI 3130 *xl* GeneticAnalyzer (Applied Biosystems). Sequence analysis was performed using Sequencher 4.8 software. The genomic DNA from 82 healthy ethnically matched control individuals was used to confirm novel mutations. A mutation is defined as any change in the sequence of a DNA molecule produced in mitosis or meiosis; an unknown change that is present in more than one of the 82 controls is considered as a polymorphism. Potential pathogenicity of genetic alterations was checked in literature, the HUS database (http://www.fh-hus.com), evolutionary conservation, splice site prediction programs (http://www.fruitfly.org/seq_tools/splice.html and http://www.cbs.dtu.dk/services/NetGene2), and SIFT (http://sift.jcvi.org).

Autoantibodies against Factor H

Nineteen patients and controls were tested for the presence of αFH by means of ELISA, as described before.[137] A positive control sample was obtained via Dr. Dragon-Durey (Paris, France). Test results were considered positive if they were above twice the standard deviation calculated from controls and samples were tested at least three times.

Deletion of the genes CFHR1 *and* CFHR3

To identify *ΔCFHR1/3*, genomic DNA was amplified by PCR using specific primers for two regions located in a 100-kb region downstream of the gene encoding complement factor H (fragment P1 and P2 in Figure 2.1A; R5 and R8 in Zipfel *et al*.[141]). Amplification of fragment P1 fails in case of *ΔCFHR1/3* (Figure 2.1B).

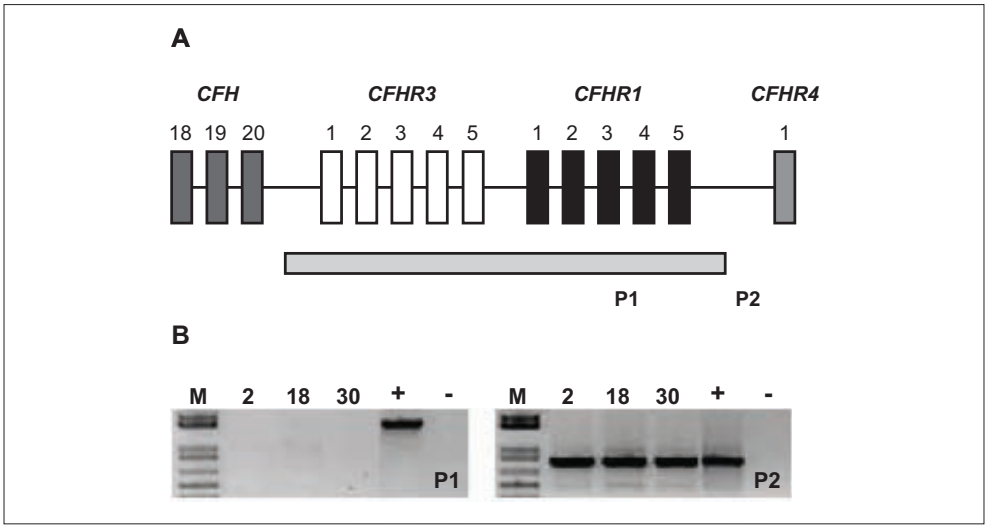

Figure 2.1. (A) Location of amplimers used for identification of deletion of *CFHR1* and *CFHR3*. The last three SCRs of *CFH,* the five SCRs of *CFHR3*, the five SCRs of *CFHR1*, and the first SCR of *CFHR4* are indicated by vertical bars. The horizontal bar indicates the location of *ΔCFHR1/3*. The position of the two amplimers (P1 and P2) is shown; amplification of fragment P1 fails in case of a homozygous polymorphic deletion of *CFHR1 and CFHR3*. (**B) PCR results of representative patients with deletion of *CFHR1 and CFHR3*.** The left electrophoresis shows the PCR product of fragment P1, the right part of the figure displays the PCR product of fragment P2. 'M' indicates marker; '+' indicates positive control; '-' negative control; numbers indicate patient subject codes.

Statistical analyses

Differences between allele frequencies of the strongly associated polymorphisms in the *CFH* gene among patients, controls, and the European population (mean from NCBI SNP database), and the presence of αFH and *ΔCFHR1/3* among patients and controls, were analyzed by calculating the 95% confidence intervals (95% CI). For analysis between patients and controls, a 95% CI that did not include zero was considered statistically significant.

Results

Genetic screening of complement factor H

The total open reading frame of the *CFH* gene was analyzed in 72 aHUS patients. Seven potential pathogenic heterozygous mutations were found in nine patients, as summarized in Table 2.1 and Figure 2.2A. Seven of these patients display sporadic aHUS, while two of them are diagnosed with the familial form of the disease. Four aberrations found have not been described before (g.-315c>t, g.IVS19+1g>a, p.Arg1203Trp, and p.Arg1206Cys); the remaining three are known disease causing mutations. Five independent mutations cluster between short consensus repeat (SCR) 16 and 20 (Figure 2.2A). Five of the detected mutations are missense mutations, one mutation affects the donor site of intron 19, causing a sequence that is not recognized as a splice site, and one mutation is located near the binding site of NFκβ, a region probably involved in the transcription of *CFH* during inflammation and infection.[79] No mutation was found in more than one of 82 healthy controls.

The presence of all three strongly associated polymorphisms in *CFH* (g.-331c>t, c.2016A>G, and c.2808G>T)[71, 79, 142] was found with a significant difference in 52.8% of the patients, in contrast to 31% of the controls (95% CI: 0.006 – 0.370; Table 2.2A). Allele frequencies of these polymorphisms (Table 2.2B and Table 2.2C) in this cohort were not significantly increased compared to those in the European population. Compared to the Dutch controls we investigated, the allele frequencies of c.2016A>G and c.2808G>T were significantly increased in patients.

Genetic screening of membrane cofactor protein (CD46)

As shown in Table 2.1 and Figure 2.2B, two heterozygous mutations were found in *CD46* in three patients with sporadic aHUS. None of the mutations were found in any of 82 healthy controls, and both mutations have been described before.[143, 144] The deletion of six nucleotides in exon 6 causes a deletion of two amino acids (Asp271 and Ser272) and was observed in two unrelated patients, whom also carried an *FI* mutation (c.454G>A and g.IVS12+5g>t, respectively). The other mutation affects the splice donor site of exon 2 and results in a splice site that is not recognized. Both mutations are located in the amino-terminal region of *CD46*, confirming the previously described importance of this region for complement regulation.[145]

Table 2.1: Characteristics of mutations found in *CFH, CD46*, and *CFI* in 17 out of 72 aHUS patients.

Exon / intron, subject code	Mutation	Effect	SCR	Known / unknown‡	Subgroup
Complement factor H					
Promotor					
40	g.-315c>t	Transcription	NA	unknown	Sporadic
Exon 9					
21	c.1198C>A	p.Gln400Lys	7	known[155]	Sporadic
Exon 19					
47	c.2850G>T	p.Gln950His	16	known[79, 147]	Sporadic
61	c.2850G>T	p.Gln950His	16	known[79, 147]	Sporadic
Intron 19					
29*	g.IVS19+1g>a	Splice site not efficiently recognized†	NA	unknown	Sporadic
Exon 23					
66	c.3607C>T	p.Arg1203Trp	20	unknown	Sporadic
68	c.3616C>T	p.Arg1206Cys	20	unknown	Familial
69	c.3616C>T	p.Arg1206Cys	20	unknown	Familial
62	c.3628C>T	p.Arg1210Cys	20	known[151, 152]	Sporadic
Membrane co-factor protein (CD46)					
Intron 2					
50	g.IVS2+2t>g	Splice site not efficiently recognized†	NA	known[144]	Sporadic
Exon 6					
23**	c.811-816delGACAGT	p.delAsp271-Ser272	4	known[143]	Sporadic
46**	c.811-816delGACAGT	p.delAsp271-Ser272	4	known[143]	Sporadic
Complement factor I					
Exon 3					
23**	c.454G>A	p.Val152Met		unknown	Sporadic
55	c.454G>A	p.Val152Met		unknown	Sporadic
Exon 9					
45	c.1019T>C	p.Ile340Thr		known[157]	Sporadic
Exon 10					
2***	c.1071T>G	p.Ile357Met		unknown	Sporadic
Exon 11					
64	c.1420C>T	p.Arg474Stop		known[70]	Sporadic
Intron 12					
1	g.IVS12+5g>t	Splice score decrease from 0.93 to 0.86†		known[71]	Sporadic
46**	g.IVS12+5g>t	Splice score decrease from 0.93 to 0.86†		known[71]	Sporadic

Patients are numbered according to an individual number. 'NA' indicates not applicable; '*' patient carrying a *CFH* mutation and a homozygous *ΔCFHR1/3* (αFH not tested) ; '**' patients carrying both *FI* and *CD46* mutations; '***' patient carrying an *CFI* mutation and αFH in combination with *ΔCFHR1/3* ; '†' according to Fruitfly and NetGene2; '‡' according to www.fh-hus.com.

Table 2.2A: Incidence and 95% CI of the three strongly associated polymorphisms in *CFH* in 72 aHUS patients and 82 controls. The percentages in the patient population are compared to the percentages in the control group by calculating 95% CI.

	Patients	Controls	95% CI (patients – controls)
Three polymorphisms	52.8%	31.0%	0.006 – 0.370*
Two polymorphisms	1.4%	8.3%	-0.133 – 0.004
One polymorphism	13.9%	22.6%	-0.206 – 0.033
No polymorphisms	31.9%	38.1%	-0.212 – 0.088

* statistically significant

Table 2.2B: Allele frequencies of the three strongly associated polymorphisms in *CFH* in 72 patients with aHUS, 82 controls, and the European population.

Genetic variant	Alleles	Patients	Controls	Eur. pop.[1]
Promotor				
g.-331c>t	C	0.583	0.690	0.857
	T	0.417	0.310	0.143
Exon 14				
c.2016A>G	A	0.681	0.792	0.720
	G	0.319	0.208	0.280
Exon 19				
c.2808G>T	G	0.681	0.804	0.838
	T	0.319	0.196	0.162

[1] Mean from SNP Database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp)

Table 2.2C: 95% CI of individual allele frequencies of the three strongly associated polymorphisms in *CFH* in 72 patients with aHUS. The frequencies in the patient population are compared to the frequencies in the control group, as well as to those in the European population.

	patients - controls	patients - Eur. pop.	controls - Eur. pop.
.-331c>t	-0.002 – 0.213	-0.847 – 1.680	-0.778 – 1.390
c.2016A>G	0.014 – 0.213*	-0.787 – 1.426	-0.686 – 1.102
c.2808G>T	0.027 – 0.222*	-0.787 – 1.426	-0.672 – 1.065

* statistically significant

Genetic screening of complement factor I

Five independent heterozygous mutational events in seven patients, all diagnosed with sporadic aHUS, were found in *CFI* (Table 2.1). Two patients carried a mutation in both *CFI* and *CD46*. Two of the observed mutations have not been described before (p.Val152Met and p.Ile357Met). Four mutations are located in the serine protease (SP) domain of Factor I [146]: two are missense mutations, one mutation introduces a premature stop codon, and one affects the donor site of intron 12 (Figure 2.2C). The aberration p.Val152Met is located in exon 3, causing a amino acid change in the (scavenger recptor cysteine rich (SCRC) domain.[27] The mutation at the donor site of intron 12 was detected in one of the 82 healthy controls; the other mutations were not found in controls.

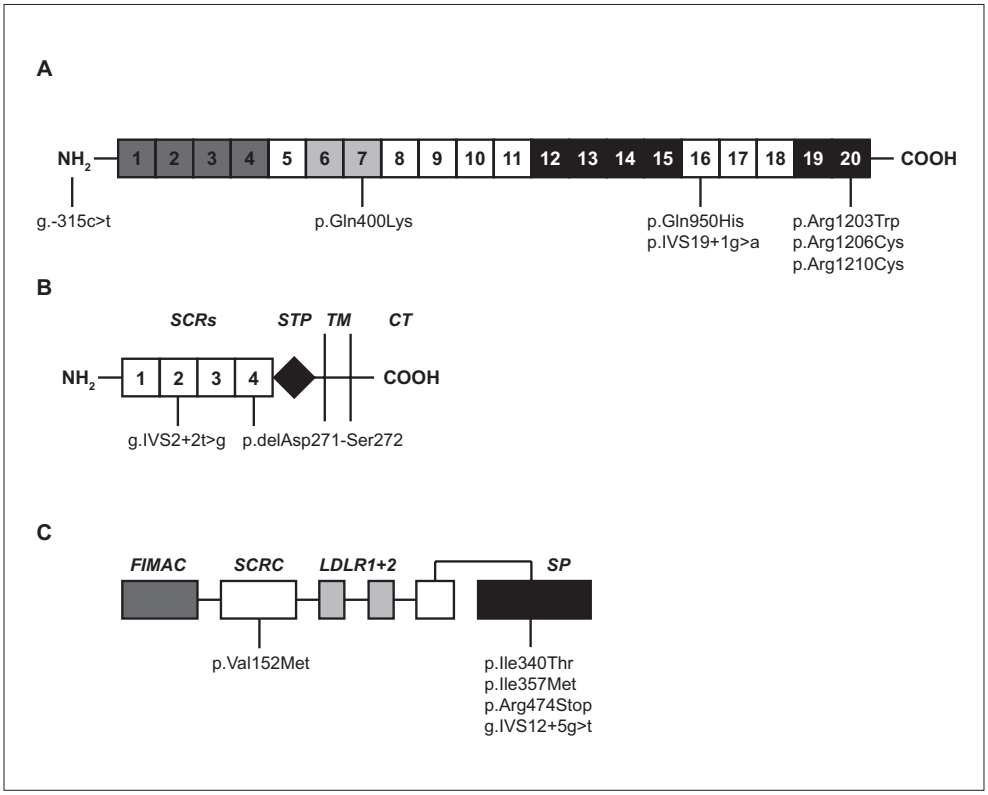

Figure 2.2. Schematic overview of the locations of described *CFH* (A), *CD46* (B), and *CFI* (C) mutations in aHUS patients of this study. SCR indicates short consensus repeat. **(A)** The white blocks indicated SCRs that are not involved in binding processes; the darker grey SCRs bind to C3b; the lighter grey SCRs bind to sialic acids and heparine sulfate; the black SCRs bind to C3b and sialic acids and heparine sulfate. **(B)** STP indicates serine-threonine-proline-rich domain; TM, transmembrane domain; and CT, cytoplasmatic tail. **C:** FIMAC indicates Factor I membrane attack complex domain; SCRC, scavenger receptor cysteine rich domain; LDLR, low-density lipoprotein receptor domain; and SP, serine protease domain.

Genetic screening of complement factor B

No potential pathogenic mutational events were found in the *CFB* gene. Several polymorphisms were observed, but none of the allele frequencies differed from the European population (data not shown).

Presence of autoantibodies against FH and ΔCFHR1/3

We found *ΔCFHR1/3* in seven of the aHUS patients, compared to three of the 82 controls; a difference that is not statistically significant (95% CI: -0.019 – 0.140). EDTA plasma of 19 patients was investigated for the presence of αFH. In total, seven patients, all children, displayed αFH, of which five had *ΔCFHR1/3* as well (Table 3). Two patients only presented αFH. In one patient (P60) with *ΔCFHR1/3*, αFH could not be detected. Of patient 29, in which *ΔCFHR1/3* was observed, EDTA

plasma was not available to test for autoantibodies; this patient carried a *CFH* mutation as well. In one patient (P2), an additional mutation in *FI* was found previously. In two controls (2/19; 10,5%), αFH were present. The difference between the patients and controls for the presence of αFH was considered significant (95% CI was 0.006 – 0.520).

Clinical information

Clinical information was obtained of all patients with a genetic aberration, except for two patients, and is shown in Table 2.3. Fourteen patients were younger than 18 years at onset of disease. Mutations in *CFH, CFI*, or *CD46* were found in patients of all ages; αFH were only found in children. Plasma C3 levels were measured in twenty patients and were low in ten. One patient died during the HUS episode. Five patients needed a transplantation (two with a mutation in *CFH,* one in *CFI* and *CD46*, two in *CFI*); in three of them (two with an *CFI* mutation, one with a *CFH* mutation) the disease recurred in the graft.

Discussion

In 31.9% of the patients (23/72), a genetic alteration in one of the investigated genes (*CFH, CFI*, *CD46*, *CFB*) or the presence of αFH was observed. An heterozygous mutational events was found in nine patients in *CFH* (12.5%), in seven patients in *CFI* (9.7%), and in three patients in *CD46* (4.2%). Six mutations were unknown genetic alterations, the remaining were known disease causing mutations. Seven of nineteen patients were tested positive for the presence of αFH (36.8%); five of these seven patients presented *ΔCFHR1/3* as well. Remarkably, three of the patients (4.2%) carried a combined mutation (*CFI* and *CD46*: 2 patients; *CFI,* and αFH, *ΔCFR1/3*: one patient). No mutations were found in *CFB*.

Seventy-one percent of the independent mutations (5/7) in *CFH* cluster between SCR 16 – 20, which confirms the previously described importance of the C-terminus of *CFH* to the pathogenesis of HUS.[79, 147-150] A mutant FH protein with loss of binding sites for C3b and polyanions shows a loss of the capability to degrade endothelial-bound C3b.[150]

The mutations p.Arg1203Trp and p.Arg1206Cys probably cause a lower binding to surface bound C3b, as they are located in the same binding site for C3b and polyanions as the p.Arg1210Cys mutation that has been described before.[151-153] These two unknown mutations are not located at a highly conserved codon (Figure 3A), but SIFT (http://sift.jcvi.org) predicts that the substitution of a

Table 2.3: Clinical features of patients with genetic aberrations in *CFH, CD46*, *CFI*, and/or the presence of αFH.

Patient	Genetic aberration	Sex	Age at presentation	Trigger	Biochemical analysis (g/l)				Treatment of first episode	Outcome	Transplantation history
					C3	C4	CFH	IF			
1	*CFI*: g.IVS12+5g>t	F	4 yrs, 1 mo	1	N/A	N/A	N/A	N/A	1	1	none
2	*CFI*: p.Ile357Met; αFH; *ΔCFHR1/3*	M	6 yrs, 6 mo	1	0.61	0.20	N/A	N/A	2	2	none
18	αFH; *ΔCFHR1/3*	F	4 yrs, 4 mo	1	0.85	0.23	normal	normal	2	3	none
21	*CFH*: p.Gln400Lys	M	1 yr, 4 mo	1	1.20	0.20	N/A	N/A	3	2	none
23	*CFI*: p.Val152Met; *CD46*: p.delAsp271-Ser272	F	30 yrs	2	0.73	0.18	N/A	N/A	2	4	1
29	*CFH*: g.IVS19+1g>a; *ΔCFHR1/3**	F									
32	αFH	M	8 yrs, 4 mo	3	0.87	0.41	N/A	N/A	4	4	none
39	αFH; *ΔCFHR1/3*	F	6 yrs, 6 mo	4	N/A	N/A	N/A	N/A	2	3	none
40	*CFH*: g.-315c>t	F	5 mo	1	N/A	N/A	N/A	N/A	2	5	none
42	αFH; *ΔCFHR1/3*	M	4 yrs, 6 mo	1	N/A	N/A	N/A	N/A	2	3	none
43	αFH	F	5 mo	1	0.72	0.15	N/A	N/A	2	2	none
45	*CFI*: p.Ile340Thr	M	1 yr, 3 mo	1	low	N/A	normal	normal	1	4	2
46	*CFI*: g.IVS12+5g>t; *CD46*: p.delAsp271-Ser272	M	13 yrs, 1 mo	1	1.00	0.19	N/A	N/A	2	5	none
47	*CFH*: p.Gln950His	M	12 yrs, 2 mo	1	0.90	0.20	N/A	N/A	2	5	none
50	*CD46*: g.IVS2+2t>g	F	37 yrs	4	1.25	0.55	normal	normal	1	5	none
54	αFH; *ΔCFHR1/3*	M	6 yrs, 5 mo	1	0.69	0.25	N/A	N/A	2	2	none
55	*CFI*: p.Val152Met	F	38 yrs	5	N/A	N/A	N/A	N/A	2	3	2
61	*CFH*: p.Gln950His	F	38 yrs	2	0.53	0.17	N/A	N/A	5	4	none
62	*CFH*: p.Arg1210Cys	M	12 yrs, 5 mo	1	N/A	N/A	N/A	N/A	1	2	none
64	*CFI*: p.Arg474Stop	F									
66	*CFH*: p.Arg1203Trp	M	39yrs	4	0.76	0.68	low	normal	5	4	2
68	*CFH*: p.Arg1206Cys	M	22 yrs	4	0.83	0.22	N/A	N/A	2	2	none
69	*CFH*: p.Arg1206Cys	F	21 yrs	6	1.75	0.4	N/A	N/A	3	4	1

Patients are numbered according to an individual number. The polymorphic homozygous *ΔCFHR1/3* was only reported in this table when occurring in combination with αFH or other genetic abnormalities in aHUS patients. For P29, the presence of αFH has not been tested (indicated with '*'). For P29 and P64, it was not possible to obtain clinical features, which is indicated with a white box. Explanation of the clinical features: *Triggers*: '1' indicates flulike; gastroenteritis, other infections; '2' unknown; '3' medication; '4' no trigger; '5' transplantation; '6' pregnancy . *Biochemical analysis*: 'N/A' indicates 'not available'; normal values C3: 0.90 – 1.80 g/l; normal values C4: 0.15 – 0.45 g/l. *Treatment*: '1' indicates supportive treatment; '2' plasmapheresis, infusion or exchange; '3' plasma and drugs acting on both coagulation cascade and immune system; '4' no treatment; '5' plasma and drugs acting on the immune system. *Outcome*: '1' indicates death; '2' complete remission; '3' chronic renal insufficiency; '4' ESRF; '5' partial remission. *Transplantation history*: '1' good renal function at 1yr; '2' disease recurrence in graft; '3' acute rejection.

positively charged, hydrophilic arginine to a neutral, hydrophobic cystine at codon 1206 will not be tolerated. The patient with the p.Arg1210Cys mutation, does not possess the FH_{tgtaat} haplotype associated with increased risk to aHUS in combination with this variation.[153] The splice site mutation at the first nucleotide after exon 19 (g.IVS19+1g>a) results in a sequence that is not recognized as a splice site by the splice site prediction programs. The p.Gln950His variation was detected previously and was reported as a mutation.[79] In another study it was declared as polymorphism.[147] We did not detect this aberration in any of our 82 controls. The amino acid located at codon 950 is highly conserved, and a change into a polar, positively charged histidine could result in functional loss of the protein. The p.Gln400Lys mutation is located in SCR 7 and surrounded by positively charged residues that are important in the binding of FH to GAGs and C-reactive protein.[154] The mutation was previously found heterozygous in the parents (first cousins) of a child whom died 15 days after birth, because of severe HUS.[155] The final mutation observed in *CFH* (g.-315c>t) is located near the binding site for NFκβ, an important transcription factor of *CFH* in case of infection and inflammation.[79] If NFκβ cannot bind properly to the gene, transcription occurs in lesser amount or does not occur at all.

As we found a statistically significant difference between the occurence of polymorphisms associated with aHUS in patients and controls, it is suggested that carrying all three disease-associated polymorphisms leads to a higher risk of developing aHUS.

Both mutations found in *CD46* were located in the extracellular SCRs with C3b binding and cofactor activity. The deletion in SCR 4 causes reduced CD46 levels and a reduced binding of approximately 50% to C3b compared to controls.[143] Reduced protein levels and reduced binding suggests that the mutant protein is less expressed on the cell membrane. The splice site mutation g.IVS2+2t>g leads in a homozygous form to a deletion of 144 base pairs and 48 amino acids, caused by a splicing of the first 45 base pairs of exon 2 onto exon 3.[144]

Eighty percent of the mutations found in *CFI* are located in the light-chain SR domain, which cleaves the alpha-chains of C3b and C4b.[156] The only variation that is not located in this region is the change from a valine into a methionine at codon 152. This mutation is located in the SCRC domain: a domain for which the function is still unknown, but probably is involved in protein-protein interactions, important in the binding of FI to FH and CD46, to ensure cofactor activity of the latter two.[146] Furthermore, codon 152 is highly conserved through evolution (Figure 2.3B). Remarkably,

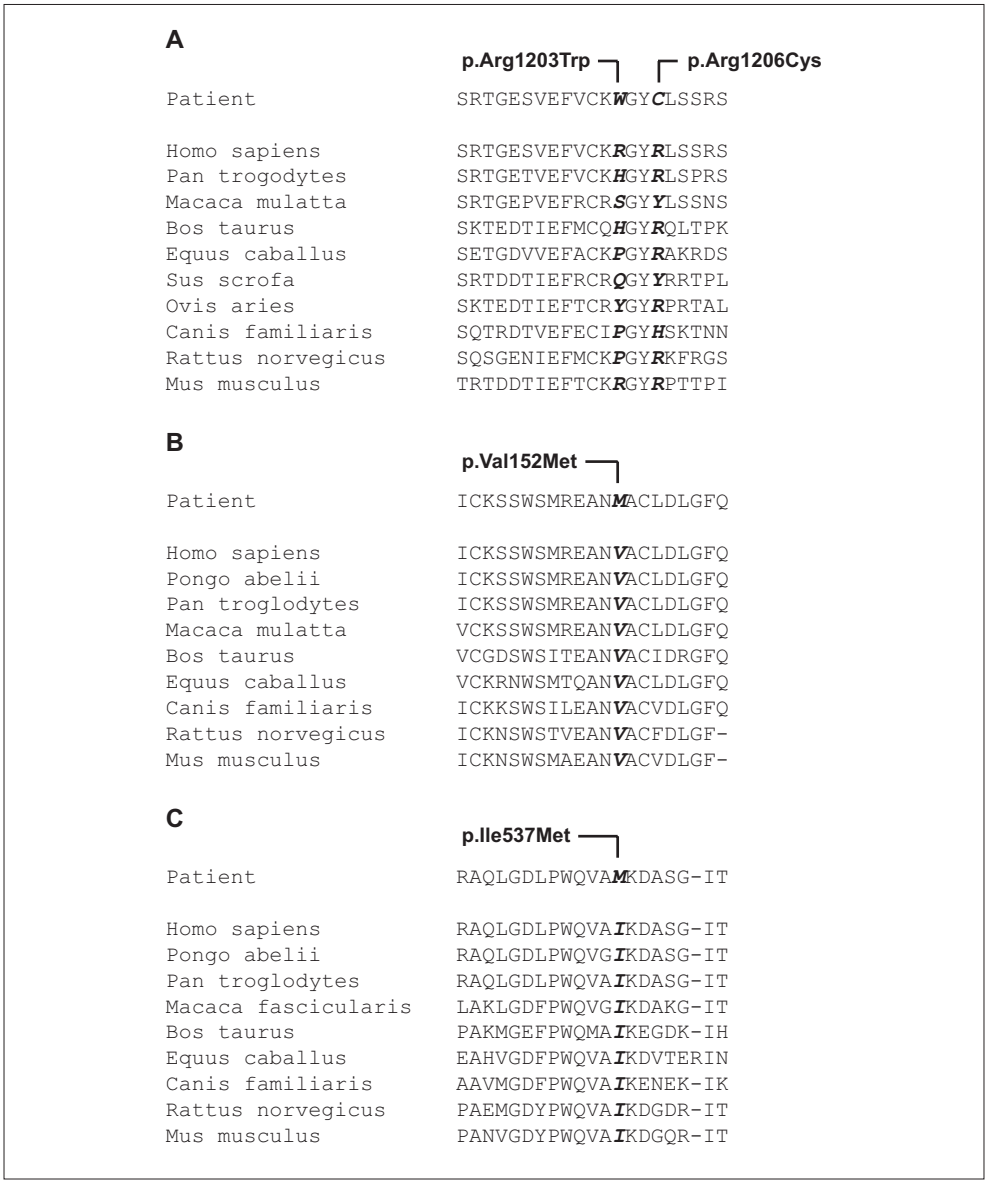

Figure 2.3: Evolutionary conservation in mammals of codons that were previously unknown for mutations. Depicted are the amino acid at codon 1203 and 1206 of FH (**A**) and codon 152 (**B**) and 357 (**C**) of FI.

this unknown change was found in two patients that were adults at onset of the disease, while the other *CFI* mutations were mostly found in children. The splice site variation found in the intron 12, resulting in a decrease of the splice score from 0.93 to 0.86 [71], was found in two unrelated patients as well as in one healthy control. This fact makes it doubtful whether the variation is a mutation or a rare polymorphism. The mutation found at codon 340 has been described before.[157] It is located at

the start of the SP domain, where isoleucine is the first amino acid of a structurally conserved region of the catalytically active domain; C3b an C4b coactivity are reduced to 0%.[158] The nonsense mutation p.Arg474Stop has been associated with heterozygous factor I deficiency and normal C3 levels.[70] Finally, the unknown mutation detected at codon 357 is located at a highly evolutionary conserved area of the protein (Figure 2.3C), indicating that a transition of isoleucine into methionine might influence the function of the protein.

Seven out of nineteen aHUS patients (of which five patients presented *ΔCFHR1/3* as well) displayed αFH (7/19; 36.8%). These five patients were all younger than seven years of age at the onset of the disease. The absence of FHR-1 and FHR-3 in the blood of a patient again seems to trigger the development of specific autoantibodies that bind to the recognition region of FH, and in this way, block the binding of FH to C3 convertases, especially in young patients.[82] It is important to test all remaining patients for the presence of αFH as well.

The mutation frequencies reported here for *CFH, CFI*, and *CD46* are slightly lower than those previously described in other cohorts, especially for *CFH*.[71, 136] This is probably due to the fact that most of our patients are diagnosed with sporadic aHUS and the frequency of mutations in these patients is lower than in patients with familial aHUS (13% - 20% and 32% - 42%, respectively).[71, 79] Differences may also be explained by regional differences between the cohorts. At this time point, already 9.7% of the patients (7/72) shows αFH. Therefore, in this aHUS group about the same amount of patients possesses αFH as in the cohort reported by Joszi *et al* (11%; 16/147).[81] The presence of *ΔCFHR1/3* in this patient population (9.7%) is slightly lower than in other cohort studies, where a deletion (both homozygous as well as heterozygous) is identified in 16 – 28% of the patients with aHUS.[81] In this study, a PCR method is used that only identifies a homozygous deletion of *CFHR1/3* and not heterozygous deletions or a hybrid *CFH/CFHR1* gene [141], which may explain the difference in results.

Remarkably, we observed several bigenic abnormalities in our aHUS patients. It is therefore important to perform routine DNA mutation analysis of all complement (regulating) genes associated with aHUS, before decisions concerning future treatment modalities like kidney donation and renal transplantation are made. Patients with a potentially pathogenic mutation or a deletion in one of the complement regulators should be discouraged to undergo a renal transplantation, especially when the mutation is located in *CFH* or *CFI*. As shown by other research groups, patients with mutations in these genes have a worse outcome after kidney transplantation than patients

with a *CD46* defect.[71, 138, 139] In our research population, only one patient has a defect in *CD46* alone; the other two patients with a *CD46* defect also possess a mutation in *CFI*. It is not yet known what the effect of αFH in combination with Δ*CFHR1/3* is on the outcome of a renal transplantation or donation, but it may be that these patients will have an increased risk of HUS recurrence in the graft and plasma exchange in combination with rituximab is recommended prior to and after transplantation.[159]

More than sixty-eight percent (49/72; 68.1%) of the patients in this study present no αFH or a genetic aberration in one of the investigated genes. Genetic disorders in other not yet examined genes involved in complement activation, like complement C3 and thrombomodulin, could have a role in aHUS as well. Further research on genes involved in complement regulation is needed to increase the understanding of the pathogenesis of the disease. Finally, this might lead to better treatment tailored to the genetic profile of the patients suffering from atypical hemolytic uraemic syndrome.

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Chapter 3

Atypical hemolytic uremic syndrome and genetic aberrations in the complement factor H-related 5 gene

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Abstract

Atypical HUS (aHUS) is a severe renal disorder that is associated with mutations in the genes encoding proteins of the complement alternative pathway. Previously, we identified pathogenic variations in genes encoding complement regulators (FH, FI, and CD46) in our aHUS cohort. In this study, we screened for mutations in the alternative pathway regulator complement factor H-related protein 5 (protein: FHR-5; gene: *CFHR5*) in 65 aHUS patients by means of PCR on genomic DNA and sequence analysis. Potential pathogenicity of genetic alterations was determined by published data on *CFHR5* variants, evolutionary conservation, and *in silico* mutation prediction programs. Detection of serum FHR-5 was performed by western blot analysis and ELISA.

A potentially pathogenic sequence variation was found in *FHR-5* in three patients (4.6%). All variations were located in SCRs that might be involved in binding to C3b, heparin, or CRP. The identified *CFHR5* mutations require functional studies to determine their relevance to aHUS, but they might be candidates for an altered genetic profile predisposing to the disease.

Introduction

Thrombotic microangiopathies are a spectrum of syndromes associated with thrombocytopenia and multiple organ failure, of which thrombotic thrombocytopenic purpura (TTP) and the hemolytic uremic syndrome (HUS) are the most common. HUS is characterized by hemolytic anemia, thrombocytopenia, and acute renal failure, and is preceded by an infection with Shiga-like producing *Escherichia coli* (*STEC*) in 90-95% of the cases.[132, 133] Atypical HUS or aHUS, which is not associated with *STEC* infection, is thought to be caused by dysregulation of the alternative complement pathway in 50-60% of the cases. Loss-of-function mutations have been identified in the regulators factor H (FH), factor I (FI), membrane cofactor protein (CD46, also known as MCP), and thrombomodulin, whilst in the complement activation proteins C3 (C3) and factor B (FB) gain-of-function mutations were found. [69-75] Recently, the presence of auto-antibodies against FH (αFH) (particularly common in the presence of polymorphic homozygous deletion of FHR-1 and FHR-3 [*ΔCFHR1/3*]) has been associated with aHUS.[81] Genetic variations are of prognostic value for the outcome of renal transplantation and kidney donation.[29]

Complement factor H-related protein 5 (FHR-5) is a member of the FH protein family that consists of six structurally and immunologically related proteins, of which FHR-5 was discovered most recently. The 65 kDa protein contains nine short consensus repeats (SCRs); SCR1 and 2 show homology with the first two SCRs of FHR-1 and FHR-2, SCRs 3-7 share significant homology with SCR10-14 of FH, and SCR8-10 are comparable to SCR19 and SCR20 of FH.[160] The protein is strongly associated with glomerular complement deposits, suggesting a role in complement regulation. It has already been shown that SCRs 5-7 can bind to heparin and C-reactive protein (CRP).[161] Furthermore, an inhibition of the C3 convertase activity in the fluid phase, a cofactor activity for FI-dependent cleavage of C3b to iC3b, and binding to C3b in a dose-dependent and saturable manner was demonstrated, although these characteristics have not been linked to specific SCRs.[160, 162] According to Monteferrante *et al*, genetic aberrations in FHR-5 might have a secondary role in the pathogenesis of aHUS.[163]

Genetic aberrations in *CFHR5* have already been associated with other glomerular diseases. In dense deposit disease (DDD or MPGN type II) three single nucleotide polymorphisms (SNPs) have been identified with significantly different allele frequencies in patients[164]; in CFHR5 nephropathy a duplication in the gene has been associated with disease.[165] Furthermore, a synonymous SNP might have a protective role in the development of age-related macular degeneration (AMD), an eye disease associated with complement dysregulation.[166] Previously, a cohort of Dutch and Belgian aHUS patients was screened for mutations in *CFH*, *CFI*, *CD46*, and *CFB*, and for the presence of αFH.

In 31.9% of the patients, complement deficiencies were found.[72] More than 4.5% of the patients carried an alteration in more than one gene. Anyhow, for more than 68% of the cohort, no cause of the disease could be identified yet. In the present study, the gene encoding FHR-5 was screened for genetic aberrations in 65 aHUS patients and FHR-5 levels were evaluated for two of the identified sequence variations.

Materials and methods

Study population

The research population consisted of 65 aHUS patients, referred to the Pediatric Nephrology Centre of the Radboud University Nijmegen Medical Centre. The patients were of Dutch or Belgian origin with a Caucasian background. In sixteen aHUS patients the familial form was identified; the remaining patients are thought to have sporadic aHUS. Permission to study DNA material was given by all patients or their parents.

Genetic analysis of gene encoding FHR-5

Genomic DNA was amplified for *CFHR5* (NCBI RefSeq NM_030787.2) by means of polymerase chain reaction; primer data are shown in Table 3.1. The primers are specific for *CFHR5*. The amplimers, including the individual exons and the splice donor and acceptor sites, were subjected to double stranded DNA sequence analysis on an ABI 3130 *xl* GeneticAnalyzer (Applied Biosystems, Carlsbad, CA, USA). Sequence analysis was performed using Sequencher 4.8 software. Note that the nucleotide and amino acid numbering begins with the start site ATG and, therefore, includes the signal peptide. Detected genetic aberrations were confirmed on a second PCR product. Genomic DNA from more than 145 healthy, ethnically matched control individuals was used to confirm sequence variations that might be potentially pathogenic. Data from the 1000 Genomes Project (http://www.1000genomes.org), in which almost all known variants with a population frequency of at least 1% are listed, and data from the Exome Variant Server (NHLBI Exome Sequencing Project (ESP), Seattle, WA; http://evs.gs.washington.edu/EVS/ [March 2012 accessed]), in which results of whole exome sequencing of almost 5400 individuals are provided, was checked for the presence of identified sequence variations as well.

Internal duplication or depletions of the first three exons of *CFHR5* and the presence of *ΔCFHR1/3* was assessed in mutated patients by multiplex ligation-dependent probe amplification (P236 A1

ARMD mix 1; MRC-Holland, Amsterdam, The Netherlands). Copy number variation was not interrogated in other patients.

Table 3.1. Oligonucleotides used to screen the coding region of *CFHR5* by means of PCR and sequencing analysis. Amplimers include individual exons and the splice donor and acceptor sites.

SCR	Forward	Reverse
1	5'-CAACCCTCCATGAACTTTGA-3'	5'-CCCCTTCAAATTATCCTCAGC-3'
2	5'-GTGATTCATCGATGTAGCTCTTT-3'	5'-TTCCAGCTCCTCTGGTCATT-3'
3	5'-TTTTCAAAGTTTTCCTTTCTTAATG-3'	5'-TTGGAACCGAAAATCAAATAAA-3'
4	5'-CACATTAAATTTGTTTCTGCAATGA-3'	5'-TCAAATTCTTGTTTCATCACTTCT-3'
5	5'-AAAGGCAATTAATTTCTAAGTCAAAA-3'	5'-AATAAAATGAGTGCTTACTCTGAAAA-3'
6	5'-TGTGGATGGAGAATGGACAA-3'	5'-AAGACCTGAATAAATGGATTGACA-3'
7	5'-TGCAGATATTTTATTGACATAATTGTT-3'	5'-TCTTGTAAAGAAGCAACAAGATCAAC-3'
8	5'-CCATTTTCCTGAAACACTACCC-3'	5'-TTGGGGTACAGTGCAACAGA-3'
9	5'-AATTATTTGAATTTCCAGACACCTT-3'	5'-GGGTTATTCTATGAAATTAGTCCAAAA-3'
10	5'-CTTAAATGCAATTTCACTATTCTATGA-3'	5'-GGCTACATAATGGCTA-3'

In silico evaluation of pathogenicity

Potential pathogenicity of genetic alterations was checked in literature, evolutionary conservation, *in silico* prediction programs SIFT (Sorting Intolerant From Tolerant; http://sift.jcvi.org/), PolyPhen-2 (Polymorphism Phenotyping v2; http://genetics.bwh.harvard.edu/pph2/), and Align GVGD (http://agvgd.iarc.fr/index.php), and in splice site prediction software (Human Splicing Finder: http://www.umd.be/HSF/; SpliceSiteFinder: http://www.genet.sickkids.on.ca/~ali/splicesite finder.html). In SIFT, a change resulting in a value below 0.05 was considered to be not tolerated and this change might therefore affect protein function. A high classification in Align GVGD (ordered from C0 to C65) indicates an aberration that is likely to interfere with protein function. For evolutionary conservation, the UCSC Genome Browser (http://genome.ucsc.edu) was used.

Statistical analysis

Differences between allele frequencies of SNPs found in patients and in the European population (Database of Single Nucleotide Polymorphisms [dbSNP]; http://www.ncbi.nlm.nih.gov/SNP/) were analyzed by calculating 95% confidence intervals (CI). Differences were considered statistically significant if the 95% CI did not include zero.

Detection of FHR-5 in serum

FHR-5 was detected in serum of HUS patients, whom were not in the acute phase of the disease, and in pooled normal human serum (NHP) by means of western blotting analysis and by enzyme-linked immunosorbent assay (ELISA) using a polyclonal rabbit anti-human FHR-5 antibody[160] (a kind gift from J. McRae, Immunology Research Centre, Melbourne, Australia) and a HRP-conjungated polyclonal swine anti-rabbit IgG antibody (Dako Denmark A/S, Glostrup, Denmark). This antibody also detects other members of the FH protein family. Serum of eighteen healthy control individuals was used to determine a control range in the ELISA; two other controls were used in the western blot.

Determination of sC5b-9 values

The soluble C5b-9 (sC5b-9) complex, also known as the fluid-phase terminal complement complex (TCC) or membrane attack complex, is increased in patients with complement activation. sC5b-9 was determined by means of ELISA in serum or plasma samples of HUS patients, whom were not in the acute phase of the disease, using a human TCC ELISA kit (Hycult Biotech, Uden, The Netherlands; catalog number HK328).

Results

Molecular genetic studies in the human gene encoding FHR-5

The total open reading frame of the *CFHR5* gene was analyzed in 65 aHUS patients. Four heterozygous sequence variations were found in six patients (Table 3.2). None of the changes were found in any of the controls. The p.Lys144Asn variation was previously described as a non-disease causing variant in a HUS family[163], is present once in the 1000 Genomes Project, and in 10 out of 5378 individuals (0.2%) of the Exome Variant Server; no RefSNP (rs) number has been assigned to this variation. Evolutionary conservation was moderate to high in all cases. Conservation within SCRs of FHR-5 was seen for p.Trp436Cys (Figure 3.1). According to SIFT and Align GVGD, protein function will be affected by all sequence variations. PolyPhen-2 predicted three of the four changes to be probably or possibly damaging; only p.Lys144Asn was predicted to be benign, probably because it is not located in a functional domain of the protein, but between SCR 2 and 3. Therefore, this aberration is considered as a previously unknown SNP. Previously, a genetic aberration was found in *CFH* in two patients; in one of these patients αFH were also found (Table 3.2). Clinically relevant data

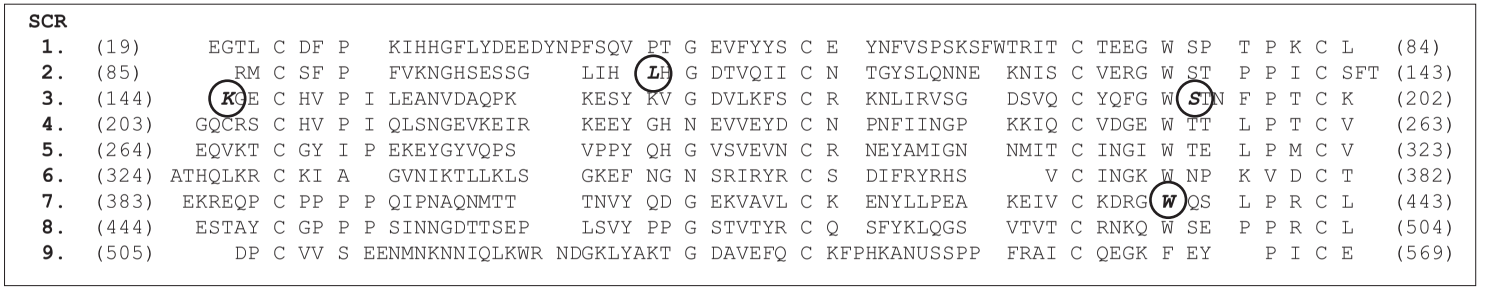

Figure 3.1. Locations of the described FHR-5 aberrations in the SCR structure of the protein. The sequences are aligned according to their conserved amino acids[160]. Amino acids coordinates are shown within brackets. The mutated amino acids are in italic and are circled.

Table 3.2. Characteristics of potential pathogenic sequence variations observed in the gene encoding human FHR-5 in our aHUS cohort. In addition, previously identified genetic and acquired complement deficiencies, and/or polymorphisms associated with aHUS are depicted.

Patient	Sequence variation	Effect	SCR	SIFT	PolyPhen-2	Align GVGD	Conservation	Controls	Previously identified complement aberrations[72]	Associated SNPs in *CFH*[79]	*CD46*$_{ggaac}$ haplotype[80]
P75	c.314T>G	p.Leu105Arg	2	N.T. (0.02)	prob. dam.	C45	moderate	0/149 (0.0%)	-	-	-
P73	c.432A>T	p.Lys144Asn[a]	-	N.T. (0.00)	benign	C65	high	0/146 (0.0%)	*CFH*: p.Arg1206Cys; αFH	3x heterozygous	homozygous
P74	c.432A>T	p.Lys144Asn[a]	-	N.T. (0.00)	benign	C65	high	0/146 (0.0%)	*CFH*: p.Arg1206Cys	3x homozygous	homozygous
P76	c.432A>T	p.Lys144Asn[a]	-	N.T. (0.00)	benign	C65	high	0/146 (0.0%)	-	3x homozygous	homozygous
P8	c.583T>A	p.Ser195Thr	3	N.T. (0.00)	poss. dam.	C55	high	0/146 (0.0%)	-	-	-
P32	c.1308G>T	p.Trp436Cys	7	N.T. (0.00)	prob. dam.	C65	high	0/152 (0.0%)	-	3x homozygous	homozygous

Patients are numbered according to an individual number. 'N.T.' indicates not tolerated; 'prob. dam.', probably damaging; 'poss. dam.', possibly damaging; 'SCR', short consensus repeat. Potential effects of the amino acid substitutions was checked using SIFT (Sorting Intolerant From Tolerant; http://sift.jcvi.org/), PolyPhen-2 (Polymorphism Phenotyping v2; http://genetics.bwh.harvard.edu/pph2/), and Align GVGD (http://agvgd.iarc.fr/index.php). For evaluation of the evolutionary conservation, the UCSC Genome browser (http://genome.ucsc.edu/) was used. Reference SNP cluster reports (RefSNP) of associated SNPs in *FH*: rs3753394, rs3753396, and rs1065489; RefSNP of associated SNPs in *CD46*$_{ggaac}$ haplotype: rs2796267, rs2796268, rs1962149, rs859705, and rs7144.

[a] Genetic aberration considered as (previously unknown) SNP.

of the patients are shown in Table 3.3. No duplications or depletions of the first three exons of *CFHR5* have been identified (data not shown).

Single nucleotide polymorphisms

Three known SNPs (rs9427662, associated with DDD[164]; rs3748557; and rs35662416, associated with AMD[166]), were identified in the coding region of *CFHR5*, but allele frequencies did not differ statistically from the European population (data not shown). Other SNPs associated with DDD or AMD were not identified. One unknown change was identified in the 3' splice site of exon 3 (c.254-5C>T), but did not result in a change at splice site level. This aberration was therefore considered as a not previously described polymorphism.

Detection of FHR-5 and sC5b-9 in serum

Serum was only available for three patients (P73, P74, and P75). FHR-5 detection by means of western blot and ELISA could therefore be performed for only two different variations: p.Leu105Arg and p.Lys144Asn. The results of the western blot and ELISA are shown in Figure 3.2 and 3.3. In the western blot, it seems that the FHR-5 level in P75 (p.Leu105Arg) may be higher than NHP; the ELISA confirms this result.

In the same three patients, sC5b-9 levels were determined to analyze complement activation. EDTA plasma samples were available for P73 and P74; for P75, only a serum sample was available. Results are shown in Table 3.2: P75 (p.Leu105Arg) shows increased sC5b-9 levels.

Discussion

Mutational screening of the gene encoding FHR-5 was performed in 65 aHUS patients. Five heterozygous genetic aberrations were identified in six patients (9.2%); one of these sequence variations (p.Lys144Asn), identified in three patients, is considered as a previously unknown SNP. Again, combined complement defects were identified among the patients (*CFHR5* and *CFH*: one patient; *CFHR5*, *CFH,* and αFH: one patient).

The sequence variation p.Trp436Cys is located in SCR7 of the protein. McRae *et al*. demonstrated that the binding sites for heparin and CRP are located within SCR5-7, although the exact binding sites are not identified yet.[162] Complement regulation at sites of tissue damage is important to prevent complement-mediated damage of host cells. Binding to the polyanion heparin on host cell

Table 3.3. Clinical features of patients with genetic aberrations in *CFHR5*.

Patient	Diagnosis	Identified complement aberrations	Sex	Age at presentation	Trigger	Biochemical analysis			Treatment first episode	Outcome	Recurrences	Transplantation history
						C3	C4	sC5b-9				
P8[a]	-	*CFHR5*: p.Ser195Thr	-	-	-	-	-	-	-	-	-	-
P32	sporadic aHUS	*CFHR5*: p.Trp436Cys	M	45 years	1	0.50 (↓)	0.40	N/A	1	1	1	1
P73	familial aHUS	*CFHR5*: p.Lys144Asn[b]; *CFH*: p.Arg1206Cys	M	22 years	1	0.83 (↓)	0.22	1.12	2	2	None	None
P74	familial aHUS	*CFHR5*: p.Lys144Asn[b]; *CFH*: p.Arg1206Cys; αFH	F	21 years	3	1.75	0.40	1.27	3	1	1	2
P75	sporadic aHUS	*CFHR5*: p.Leu105Arg[b]	F	8 years	4	N/A	N/A	18.28 (↑)	4	1	1	1
P76	sporadic aHUS	*CFHR5*: p.Lys144Asn	F	18 years	4	0.48 (↓)	0.19	N/A	2	1	1	1

Patients are numbered according to an individual number. For P8 it was not possible to obtain clinical features. Explanation of the clinical features: 'Triggers': 1 indicates no trigger; 2 flulike, gastroenteritis, other infections; 3 pregnancy; 4 unknown. 'Biochemical analysis': (↓) indicates low values; N/A not available; normal values C3: 0.90 – 1.80 g/l; normal values C4: 0.15 – 0.45 g/l; normal levels sC5b-9: <1.0 AU/ml. 'Treatment': 1 indicates plasma, supportive treatment and drugs acting on immune system; 2 plasmapheresis, infusion or exchange; 3 plasma and drugs acting on both coagulation cascade and immune system; 4 unknown. 'Outcome': 1 indicates ESRF; 2 partial remission; 3 complete remission. 'Transplantation history': 1 indicates disease recurrence in graft; 2 good renal function at 1 year.

[a] Not possible to obtain clinical features for this patient; [b] Genetic aberration considered as previously unknown SNP.

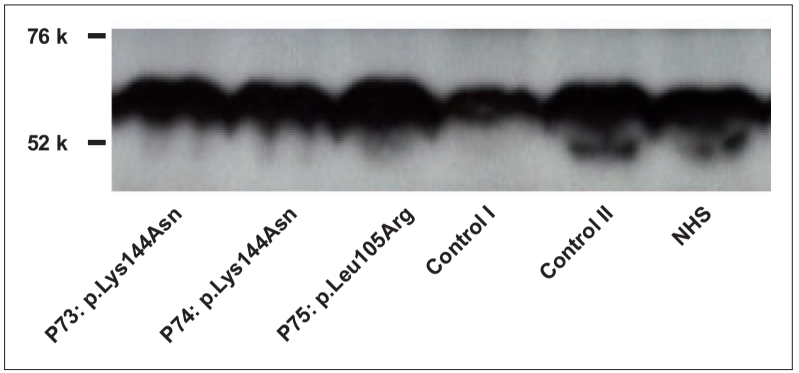

Figure 3.2. Detection of FHR-5 in serum samples of mutated patients by western blot. Western blot of serum samples of three patients, two controls, and pooled human serum with polyclonal anti-FHR-5 antibody for detection of FHR-5. Molecular mass marker proteins are indicated on the left.

membranes is probably important in positioning of FHR-5 on host cells to regulate complement activity at the site of tissue damage. In this aberration, the nonpolar amino acid tryptophan is changed into a polar cysteine, which could influence heparin binding. Furthermore, introduction of a cysteine at codon 436 might result in new disulfide bonds and therefore in different protein folding.
The remaining variations are located in SCR2 and SCR3, which are for 37% homologous to SCR6 and SCR7 of FH that show binding capacity to C3b and heparin as well.[167] A recombinant FHR-5 protein fragment containing SCR1-4, however, did not show CRP and heparin binding, while a SCR3-7 fragment did show binding capacity. Therefore, the involvement of SCR3 and 4 in heparin and CRP binding cannot be ruled out, but alone they are not sufficient enough. Binding to C3b has not been localized to specific SCRs of FHR-5 yet, but as C3b binding sites are present in SCR6 and 7 of FH, they might be present in SCR2 and 3 of FHR-5 as well.
All variations might result in decreased complement regulation. Ongoing activity of the alternative complement pathway will lead to an increased formation of membrane attack complex and to increased attraction of leukocytes to the site of endothelial damage, resulting in local thrombosis and inflammation in the glomerular vessels, and eventually to thrombotic microangiopathy and HUS.

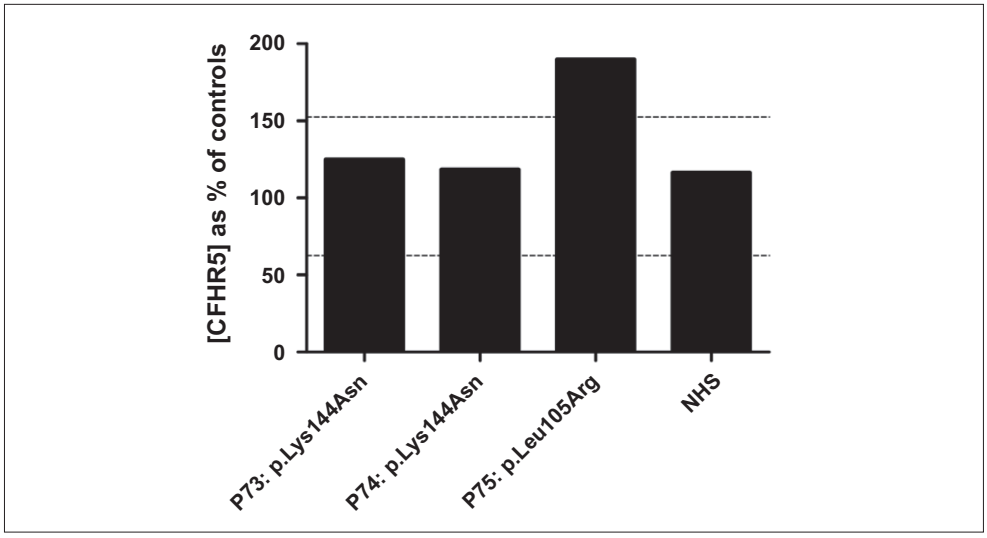

Figure 3.3. Detection of FHR-5 in serum samples of mutated patients by ELISA. FHR-5 antigen levels measured by ELISA, expressed as percentage in respect to the mean concentration in the control group of 18 healthy individuals (P73: 124.8%, P74: 118.5%, P75: 189.7%, pooled human serum: 116.7%). The range within the controls is shown (64% - 152%).

The p.Lys144Asn aberration has been described before in two affected sisters and their healthy mother in an aHUS family.[163] Another affected family member did not possess this aberration, and therefore it did not seem to be the primary cause of HUS in that specific family, but it was not found

in 80 screened control individuals. We identified this sequence variation in three aHUS patients, but not in any of 146 control individuals, while it is present once in the 1000 Genomes Project and in 0.2% of the individuals in the Exome Variant Server. We do agree that p.Lys144Asn does not seem to be the primary cause of the disease, as P73 and P74 also carry a pathogenic aberration in *FH* (p.Arg1206Cys)[72] and the variation did not appear to influence protein levels in serum. The three remaining aberrations could still be the primary cause of aHUS in our patients: they were not found in our controls, all prediction programs predicts them to be damaging to protein function, and no other genetic variations were identified in these patients yet (Table 3.2). The p.Leu105Arg variation showed a slight increase in FHR-5 protein levels. The reasons for this are not clear. We do see that sC5b-9 levels are altered in the serum of P75, which indicates a increased complement activation. It is known that sC5b-9 levels are higher in serum than in plasma, but even in serum of control individuals, levels were not higher than 3.5 AU/ml (data not shown). Although no other genetic aberration has been identified in this patient, we cannot conclude that the p.Leu105Arg variation is indeed the cause of this increase. The exact functions of FHR-5 are still not known and no functional tests are available to investigate this, yet.[168]

It has been shown that FHR-5 has a high affinity for heparin on host cell surfaces, even higher than FH.[162] On the other hand, its cofactor and convertase inhibitory activity is lower than FH, which suggests a specific complement regulation at the site of glomerular endothelial cell surfaces instead of in the fluid phase. This is emphasized by the association with glomerular immune deposits. The question remains why a dysregulation of the complement system at the cell surface due to variations in *FHR-5* can result in both FHR-5 nephropathy, a C3 glomerulonephritis, and aHUS, a thrombotic microangiopathy. One explanation might be the different locations of the genetic aberrations; in CFHR5 nephropathy, a duplication of SCR1 and SCR2 is seen, while in aHUS, variations are also identified in SCR3-9. Anyhow, as the exact biological role of FHR-5 in complement regulation is still not fully understand, a precise answer cannot be given.

The outcome of plasmapheresis and renal transplantation is correlated with the type of complement mutation.[29] Patients with aberrations in complement proteins produced in the liver, like FH and FI, have a worse outcome after renal transplantation than patients with a mutation in the membrane-bound CD46. This could be the case in patients with a change in *CFHR5* as well, as this soluble protein is produced in the liver, although we do see that one of our transplanted patients (P74), with both a mutation in *CFH* and a previously unknown SNP in *CFHR5*, still had good graft function after

one year. Three other patients had a recurrence of the disease in the graft within one year after transplantation. Eculizumab, a monoclonal anti-C5 antibody that has positive effects in the treatment of aHUS patients [169], might also be a treatment option for aHUS patients with *CFHR5* aberrations.

The prevalence of potentially pathogenic sequence variations in *CFHR5* in our cohort (4.6%) are higher than in two other screened aHUS cohorts. Monteferrante *et al*. identified four different genetic alterations in 17.7% (8/45) of their cohort, but three of these variations were found in 1.3% of the controls as well.[163] In an American cohort, a novel mutation was identified in *CFHR5* in 2/144 (1.4%) aHUS patients.[76] The differences between these cohorts and ours might be explained by regional differences.

In our research population of 65 aHUS patients, the genes encoding FH, FI, CD46, FB, and FHR-5 were have been screened. In 38.5% (25/65) of the patients, at least one sequence variation was identified in one of the complement genes. Five of these patients (7.7%) carried bigenic aberrations. Atypical HUS is now considered to be driven by a combination of genetic susceptibility factors (mutations and associated polymorphisms) and an environmental trigger. In the remaining patients, no genetic aberrations have been found yet, and therefore, further research on genes involved in the complement system and the coagulation cascade is needed to increase the knowledge about the pathogenesis of aHUS. Understanding how sequence variations influence aHUS susceptibility will require detailed functional analysis so that rare sequence variations are not mistaken as disease-associated changes. As the exact biological role of FHR-5 in HUS and other renal diseases is not known yet[168], the investigation of the significance of identified sequence variants to disease pathogenesis still has to be established.

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Chapter 4

A novel C3 mutation p.Lys65Gln in aHUS affects complement factor H binding

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Abstract

Atypical hemolytic uremic syndrome (aHUS) is associated with mutations affecting complement proteins and regulators and with autoantibodies against complement factor H. Approximately half of the aHUS patients progress to end-stage renal disease. DNA analysis of the risk factor genes is important for prognosis of aHUS recurrence after renal transplantation. Mutational screening of *C3*, encoding the central complement component, was performed by Sanger sequencing in 70 aHUS patients. Mutated and wild type recombinant C3b proteins were produced and their affinity to complement factor H (FH) was analyzed by ELISA. A single novel missense change p.Lys65Gln in C3 was found in three aHUS patients. The alteration leads to decreased binding of C3b to FH in vitro. All three patients acquired the illness as adults and had a first aHUS episode after renal transplantation or suffered recurrence of the disease after transplantation. The p.Lys65Gln variation is likely to be associated with aHUS after kidney transplantation and, therefore, might be an important prognostic factor.

Introduction

The hemolytic uremic syndrome (HUS) is characterized by hemolytic anemia, thrombocytopenia, and acute renal failure.[170] In most cases, HUS is preceded by infection with Shiga-like toxin producing *Escherichia coli* (*STEC*). Five to 10% of all HUS patients acquire the disease without being infected with *STEC*. These atypical HUS (aHUS) patients have a poor prognosis with up to 50% of cases progressing to end stage renal disease (ESRD) and up to 25% of lethal outcomes in the acute phase.[37] Furthermore, HUS can occur due to a variety of causes, including non-enteric infections (*Streptococcus pneumoniae*), use of medication, and pregnancy.[171-173]

The aHUS etiology has been linked to ongoing alternative complement pathway activation. In this alternative pathway, complement component C3 is spontaneously activated at a very low rate to form C3b. The C3b is able to attach to the surfaces of pathogens and host cells, where it binds complement factor B (FB), which in turn is cleaved by complement factor D (FD). The resulting C3bBb, or C3 convertase, cleaves and activates C3 leading to amplification of the complement cascade, to the formation of a membrane attack complex, and, eventually, to cell lysis. At the surface of the normal host cells, C3b is cleaved by complement factor I (FI), while complement factor H (FH), membrane cofactor protein (CD46/MCP), and complement receptor type 1 act as cofactors. In addition, at the surface of the normal host cells, C3 convertase can be dissociated by regulators.[66] Mutations affecting FH, FI, CD46, C3, FB, thrombomodulin, and the presence of autoantibodies against FH are associated with aHUS pathogenesis.[69-71, 73-75, 80, 81] Complement deficiencies are identified in 50-60% of aHUS patients.[29, 95] In particular, new C3 mutations affecting C3 convertase in a gain-of-function manner were recently described.[174, 175]

Etiological analysis of patients with aHUS is very important, especially in renal transplantation, which is frequently required in this patient group. For example, patients that carry mutations in genes encoding FH or FI are at higher risk of the disease recurrence in the graft (70-90%), whereas such probability is much lower for the aHUS patients carrying CD46 mutations (20%).[29, 95] Previously, we reported prevalence of mutations in FH, FI, CD46, and FB, and autoantibodies against FH in Dutch/Belgian aHUS cohort.[72] In this study we report C3 variations found in our aHUS patients.

Methods

Study population

The research population consisted of 70 aHUS patients (age 2 months to 52 years at onset of the disease), referred to the Pediatric Nephrology Department of the Radboud University Nijmegen Medical Centre. All patients were of Dutch or Belgian origin and diagnosed with non-*STEC*-HUS. In 15 patients from 10 families, the familial form of aHUS was identified; the other 55 patients were diagnosed with sporadic aHUS. Informed consent of all patients or their parents was obtained before the DNA analysis. The missense *C3* alterations found in patients were also analyzed in genomic DNA from 100 healthy ethnically-matched control individuals.

Sequence analysis of the C3 gene

Genomic DNA was isolated from peripheral blood leukocytes as described by Miller *et al*.[140] Fragments of the *C3* gene (NCBI mRNA RefSeq NM_000064.2, genomic RefSeq NG_009557.1)[176] were amplified from genomic DNA by means of PCR. Primer sequences are available upon request. The obtained PCR products included DNA sequences of the 41 individual exons, flanked by the splice donor site and the splice acceptor site. The amplimers were subjected to double-stranded DNA sequence analysis on an ABI 3130 *xl* GeneticAnalyzer (Applied Biosystems). Sequence analyses were performed using Sequencher 4.8 software (Gene Codes). Sorting Intolerant From Tolerant (SIFT) (http://sift.jcvi.org/)[177] and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/)[178] mutation analysis algorithms were used to access potential pathogenicity of C3 changes on protein level. The SIFT scores were obtained by submitting C3 protein sequences of *Homo sapiens*, *Bos taurus*, *Sus scrofa*, *Canis familiaris*, *Rattus norvegicus*, *Mus musculus*, and *Xenopus tropicalis* to the program. Substitutions with scores below the threshold of 0.05 are considered intolerant by SIFT and are likely to affect protein function.

Recombinant C3b production

A DNA fragment encoding mature wild type C3 protein was cloned into the PCR4-TOPO (Invitrogen); the c.193A>C (p.Lys65Gln) and c.481C>T (p.Arg161Trp) sequence variations were introduced using the QuikChange method. Subsequently, the C3 variants were subcloned into a modified pUPE expression vector (U-Protein Express BV). The wild type and mutant C3 constructs were expressed in HEK293-E cells in the presence of furin to insure correct C3 processing [179]. After three days of expression, medium was collected and centrifuged (1000g, 15 minutes, 4°C), supernatant was used

in experiments. The expression levels of C3 in the medium were 2-7 µg/ml. C3b was generated from C3 using FB and FD. The medium samples containing 1 µg/ml C3 were incubated with 1.8 µg/ml FB and 0.13 µg/ml FD (both from Complement Technology) for 2 hours at 37°C. Cleavage of C3a was verified by SDS-PAGE.

Binding affinity assay

The binding affinity assay was done in ELISA setting. Wells of the ELISA plates were coated with 1 µg/ml of purified FH (Calbiochem). The coated wells were incubated with medium samples containing 125, 250, 500, or 1000 ng/ml of wild type or mutant C3b and the presence of C3b was detected using horseradish peroxidase (HRP)-conjugated goat antibodies against C3 (MP Biomedicals).

Statistical analyses

The statistical significance of allelic frequencies was analyzed using the two-tailed Fisher's exact test. The statistical analysis of the binding affinity assay results was performed using two-way ANOVA. The differences with $p<0.05$ were considered statistically significant.

Results

DNA alterations identified in C3 in three aHUS patients

The total open reading frame of the *C3* gene was analyzed in 70 patients with aHUS. A novel missense DNA change c.193A>C, leading to a p.Lys65Gln substitution, was identified in three patients in heterozygous form (Figure 4.1a, Table 4.1).

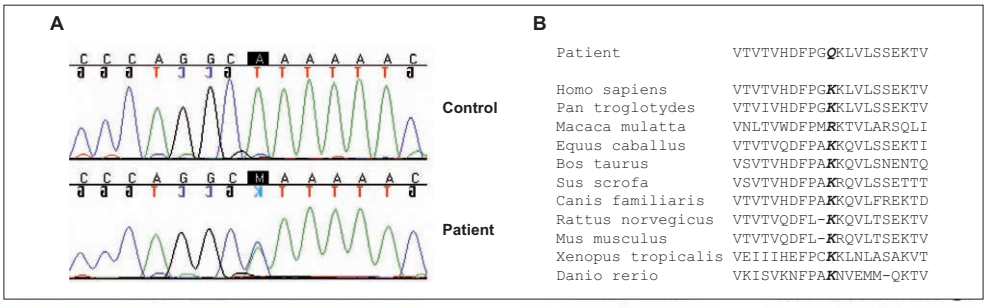

Figure 4.1. Novel sequence variation found in three aHUS patients. (A) Sequencing results of healthy control (upper panel) and patient (lower panel). Location of c.193A>C is indicated by black box. **(B)** Sequence alignment of the C3 protein regions from various species containing Lys65, altered in aHUS patients. Altered amino acid position is marked by black box and its number is indicated.

All of the patients had a sporadic form of the disease and did not carry mutations affecting FH, FI, CD46, FB, or thrombomodulin, or were positive for autoantibodies against FH. The novel change was not found by us among 100 healthy controls, nor is it reported in dbSNP. It is also not reported in NHLBI Exome Sequencing Project (ESP), which carries whole exome sequencing data from over 5000 human exomes (http://evs.gs.washington.edu/EVS/). The p.Lys65Gln alteration affects a conserved residue (Figure 4.1b). It has a SIFT score of 0.00 and is predicted as probably damaging by PolyPhen-2, indicating intolerance and a possible impact on the C3 structure and/or function. We also encountered a recently described p.Arg161Trp (referred to by the authors as p.Arg139Trp, while not counting the signal peptide) in 11 patients in heterozygous form.[175] Interestingly, although this change was not found by us among 100 controls, we did detect it in three healthy parents of aHUS patients, while the patients themselves did not possess the change. The prevalence of this polymorphism among aHUS patients was significantly higher (p=0.01) than among the screened healthy individuals.

Table 4.1. Clinical data available for patients carrying p.Lys65Gln.

Patient	Gender	Age at onset	C3 levels in acute aHUS phase[a]	Transplantation history	Outcome
1	M	40	0.73-0.95	aHUS after transplantation[b]	Partial recovery
2	F	18	0.48-0.76	aHUS after transplantation[c] and aHUS recurrence in second graft	ESRD
3	M	45	0.5	Transplantation after aHUS and aHUS recurrence in the graft	ESRD

'aHUS' indicates atypical hemolytic uremic syndrome; 'ESRD', end-stage renal disease.

[a] C3 normal values: 0.70–1.50 g/L

[b] Kidney transplantation related to thrombotic microangiopathy as a result of malignant hypertension

[c] Kidney transplantation related to rapidly progressing glomerulonephritis

The aberration p.Lys65Gln decreases C3b binding to FH in vitro

The altered amino acid is located in the part of the *C3* gene encoding C3b, at the interface of the C3b and FH domain 4 (Figure 4.2a).[180] Lysine, at position 65 in C3b, is in direct contact with the FH glutamate at position 245 (Figure 4.2b). These C3b and FH residues are forming a salt bridge, a relatively weak ionic bond between positively charged lysine and negatively charged glutamic acid. Replacement of a lysine with a glutamine might, therefore, weaken the interaction between FH and C3b. To test this hypothesis, recombinant C3 protein, carrying p.Lys65Gln was produced and cleaved using FB and FD to yield C3b. Binding of the recombinant mutant and wild type C3b to purified FH was compared in ELISA setting (Figure 4.3a). The p.Lys65Gln change resulted in statistically

significant ($p<0.001$) decrease in FH binding when C3b concentration reached 1000 ng/ml. This finding indicates that the DNA alteration leads to the weaker affinity of C3b to FH.

In contrast to the previously reported data [175], in the similar experiment we also observed a weaker binding for C3b variant carrying p.Arg161Trp (Figure 4.3b). Although this decrease is less pronounced than that of p.Lys65Gln, it is statistically significant ($p<0.05$).

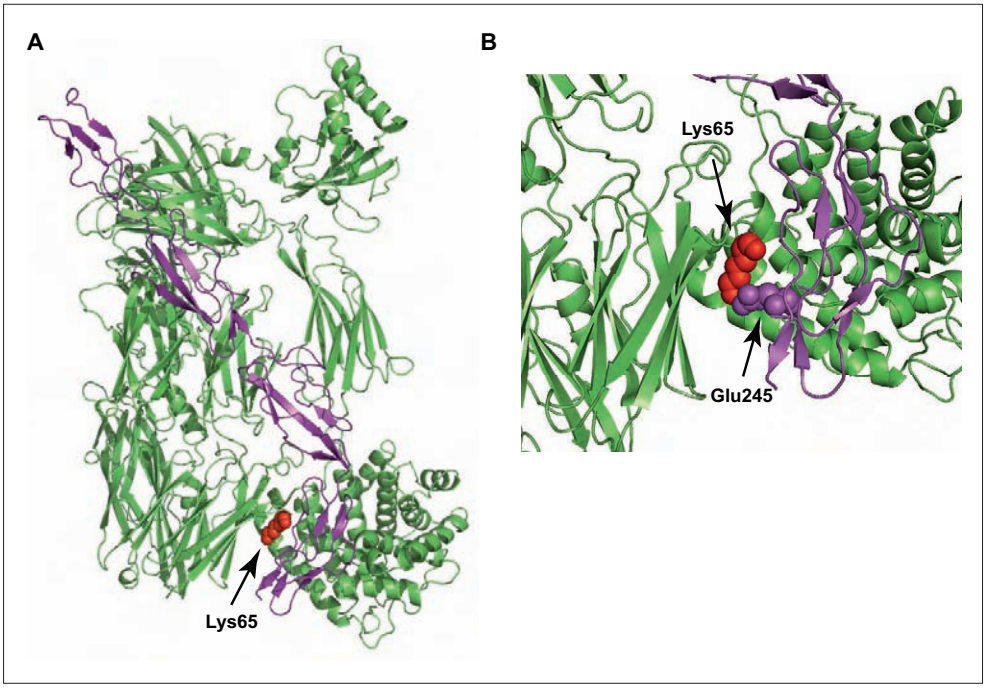

Figure 4.2. Location of Lys65 amino acid altered by the missense mutation in the aHUS patients. C3b domains are colored in green and FH domains are shown in purple. Amino acid residue mutated in aHUS patients is indicated by red spheres. **(A)** Structure of C3b in complex with FH domains 1 - 4.[25] **(B)** Enlarged image showing direct interaction of Lys65 residue of C3b with Glu245 residue of FH. The images were generated using PyMol.

The p.Lys65Gln alteration is found in adult patients with aHUS in a kidney graft

All patients carrying p.Lys65Gln were adults at the time of the first aHUS episode.

The first patient was initially diagnosed with thrombotic microangiopathy as result of malignant hypertension in 2003. Hemolytic anemia and thrombocytopenia were not found, the thrombotic microangiopathy (TMA) diagnosis was made based on the biopsy results. The patient was referred to hemodialysis and later received a living-donor kidney from his sister. Five months later, the patient developed thrombotic microangiopathy combined with the declining renal function. Serum C3 levels

were at the lower border of the normal range (Table 4.1) and the patient was diagnosed with aHUS. Incomplete recovery of the kidney function after this episode was achieved.

The second patient initially suffered from rapidly progressing glomerulonephritis (RPGN) of undefined etiology in 2003. The laboratory findings show no hemolytic anemia or thrombocytopenia, renal biopsy was not performed, therefore presence of TMA was not determined. The patient received a living-donor kidney transplant from her father. She developed aHUS in this renal graft and also in the next cadaver kidney transplant. In the third cadaver kidney transplant, the patient developed acute tubular necrosis and was referred to hemodialysis. Eventually the patient died due to gram-negative septic shock.

The third patient developed TMA, diagnosed by renal biopsy, in combination with low C3 levels in serum. The patient received a kidney transplant, six months later the aHUS recurred in the graft.

Interestingly, in all three patients aHUS (re)occurred after kidney transplantation.

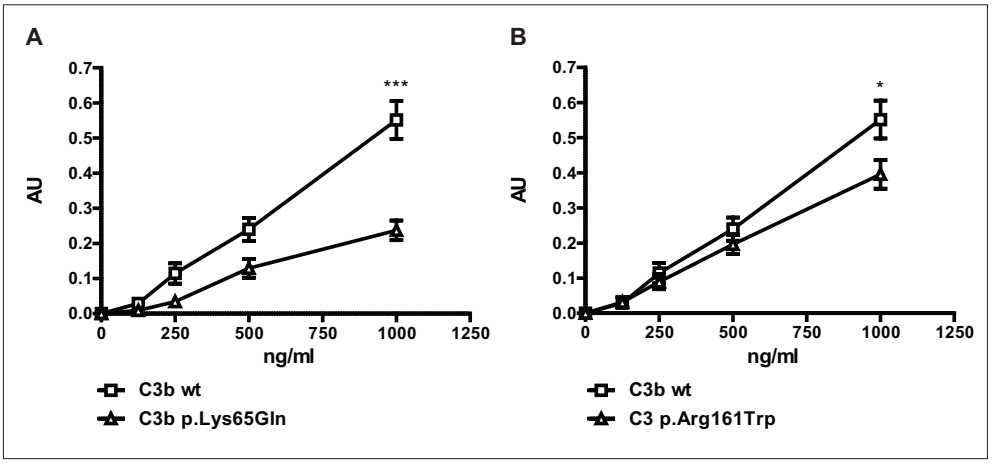

Figure 4.3. C3b binding affinity to FH. ELISA plates were coated with purified FH, after that the wells were incubated with various concentrations of the recombinantly produced wild type and p.Lys65Gln **(A)** or p.Arg161Trp **(B)** C3b variants. Binding of the C3b variants was detected using antibodies against C3. ELISA results are expressed in arbitrary units (AU). The data represent four independent experiments and is presented as mean±SE. Significant differences according to ANOVA with $p<0.001$ (***) and $p<0.05$ (*) are indicated.

Discussion

In this study, a novel missense sequence variation c.193A>C was found leading to a p.Lys65Gln substitution in the *C3* gene. The mutation alters a highly conserved amino acid (Figure 4.1b). All of the analyzed species carry a lysine at position 65, except for *Macaca mulatta*, where its place is taken by an arginine, which is, similar to lysine, a positively charged hydrophilic amino acid. On the

contrary, a glutamine, found at this position in aHUS patients has a neutrally charged side chain. As shown by our data, the replacement of lysine with glutamine compromises C3b-FH interaction. It might lead to the decreased rate of C3b cleavage by FI and decreased dissociation of C3 convertase by FH decay-acceleration activity *in vivo*. Inefficient complement inactivation at the cell surface would result in damage of endothelium of the glomeruli.

Further experiments should be considered for future analysis to increase impact of functional role of mutation, such as measurement of complement activation products in serum of controls and patients, carrying the mutation. Furthermore, complement deposition on human glomerular microvascular endothelial cells and human umbilical vein endothelial cells from patient and control serum can be compared.

Clinical data were available for all three patients. All of the patients acquired aHUS in renal transplants. This finding is important, because it indicates that the p.Lys65Gln substitution in C3 might be associated with poor prognosis in renal transplantation.

The previously described aberration p.Arg161Trp was found in 11 patients and in three healthy parents of other aHUS patients that themselves did not possess the change. The incidence of the p.Arg161Trp substitution is significantly higher in the aHUS group than among the healthy individuals. This indicates that p.Arg161Trp is rather an aHUS-predisposing single nucleotide polymorphism than an aHUS-causing mutation. Furthermore, our data indicate a significant weakening of the C3b-FH interaction by p.Arg161Trp. This weakening was not observed previously.[175] Roumenina *et al*. used a concentration range 0-300 ng/ml of C3b, while in our studies we used a broader C3b concentration range and observed a significant weakening of FH binding at 1000 ng/ml C3b. Our findings provide new insight into the pathogenicity mechanism of p.Arg161Trp, a strongly predisposing aHUS polymorphism.

Previously, we reported genetic aberrations found in *CFH*, *CFI*, *CFB* and *CD46* and presence of autoantibodies against FH in our aHUS patient cohort.[72] In this study, we described a potentially pathogenic p.Lys65Gln mutation in the *C3* gene in our patients. Moreover, in 11 patients we identified a p.Arg161Trp polymorphism, which is strongly predisposing to aHUS. In total, the prevalence of C3 changes in Dutch/Belgian aHUS cohort is 20% (14/70). Together with the previously reported findings, 48.6% (34/70) of patients in our aHUS cohort display potential disease causing alterations in genes encoding complement (regulating) proteins.

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Chapter 5

The application of whole exome sequencing in patients with familial atypical hemolytic uremic syndrome

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In preparation

Abstract

Atypical hemolytic uremic syndrome (aHUS) is a rare, but severe disease that has a heterogeneous genetic background. Mutations in several genes encoding (regulating) proteins of the alternative complement pathway have been associated with the disorder. Due to the heterogeneity seen in aHUS, making a genetic diagnosis via regular methods is relative expensive, labor intensive, and time consuming. Whole exome sequencing (WES), in which all coding exons of a genome can be studied simultaneously, may be an attractive alternative strategy for mutation scanning in aHUS patients.

To investigate the clinical utility of WES for genetic testing in aHUS patients, exomes of ten patients diagnosed with familial aHUS were captured and enriched using the 50MB Agilent's SureSelect human exome v4 enrichment kit (Agilent, Santa Clara, CA, USA). DNA of one of the patients served as a positive control (tested for a *CFI* mutation).

More than 47000 sequence variations were identified per patient. On average, ~230 private variants were located in an exon or canonical splice site. All were absent in the dbSNP database or present in less than 1% of an in-house database. Overall, 10x coverage, i.e. >10 reads anywhere in an exon, of associated aHUS genes was 87%. SCR19 and 20 in *CFH* were not covered at all. The *CFI* mutation in the control patient was detected.

As one of the more prominent hotspots for mutations in aHUS patients (SCR19-20 in *CFH*) is not sequenced, the sole application of WES is not possible without any adjustments as it will lead to a lower diagnostic yield compared to current diagnostic testing. However, next generation sequencing (be it in an exome sequencing or targeted gene panel approach) in combination with conventional amplicon based sequencing of the missing *CFH* region and the deep intronic SNPs of aHUS associated haplotypes can be used to maintain or even improve current diagnostic yields in a more efficient manner.

Introduction

The hemolytic uremic syndrome (HUS) is a rare thrombotic microangiopathy, characterized by hemolytic anemia, thrombocytopenia, and acute renal failure.[170] In more than 90% of the cases, the disease is preceded by an infection with Shiga-like toxin producing *Escherichia coli* (*STEC*).[133] About 5% to 10% of the cases are not associated with *STEC*; these atypical HUS (aHUS) cases have a much poorer prognosis, with progression into end-stage renal disease in up to 50% of the cases and a mortality of 25% during the acute phase of the disease.[29]

In recent years, the role of complement regulation in the pathogenesis of aHUS has made important progress. The first description of a possible association between the hemolytic uremic syndrome and the alternative pathway of the complement system was made in the 1970s and 1980s, when low C3 levels, but normal C4 levels were identified in HUS patients.[64] The first genetic link was made in 1998 by Warwicker *et al*., whom identified heterozygous mutations in complement regulator factor H (protein: FH; gene: *CFH*) after linkage analysis in patients with sporadic and familial HUS.[68] Since then, seven other genes have been associated with the atypical form of HUS (*CD46*, *CFI*, *CFB*, *C3*, *THBD*, and recently *DGKE* and *PLG*), all involved in the alternative complement pathway or in the coagulation system.[70, 73-75, 83, 85, 143] Autoantibodies against factor H (αFH), which can bind to epitopes of FH that are involved complement regulation, have been identified in patients as well.[137] Knowing which genetic variations are present in patients diagnosed with aHUS is important, as this knowledge can be of prognostic value for the outcome of kidney transplantation, as well as for decision making in donating a kidney to a family member, and for the successful outcome of treatment with the complement inhibitor eculizumab[83].

A large heterogeneity is seen in aHUS: already eight genes have clearly been associated with the disease, next to predisposing SNP haplotypes in *CFH* and *CD46*.[80] Furthermore, an incomplete penetrance is seen, as healthy family members can have the same mutation as a patient. A combination of mutations and at risk haplotypes might be needed for the disease to develop[92], but probably a trigger for complement activation is needed as well.

The current strategies to get to a genetic diagnosis are using standard amplicon based Sanger sequencing methods and are labor intensive, costly, and time consuming when compared to next generation sequencing (NGS) based strategies: to screen the entire coding sequence of all currently associated genes, 184 exons (>30 kB) need to be sequenced. Although this diagnostic route detects causative mutations in 50-60% of the cases, still over 40% of all aHUS patients cannot be explained.[29] Whole exome sequencing (WES) is a DNA sequencing approach in which in principle all

the coding sequences of the genome can be studied in a single experiment.. This approach is of great diagnostic value in genetically heterogeneous diseases, as shown in more common diseases as intellectual disability[181] and hereditary blindness[182], but also for rare diseases like mitochondrial disorders[183]. WES might be an attractive alternative routing for mutation scanning in aHUS patients as well. Apart from sequencing all coding regions of the associated aHUS-genes WES would also allow the discovery of new aHUS genes in mutation negative patients.

Materials and methods

Patient population

Ten patients diagnosed with familial aHUS (two or more family members with aHUS) were included in this study, of which genomic DNA was extracted from blood according to established protocols. The associated genes *CFH*, *CFI*, *CD46*, *C3*, and *CFB* were screened before by Sanger sequencing, and in eight patients the aHUS associated polymorphism rs147859257 in *C3* (p.Arg161Trp)[94, 175] was identified. This variation is also present in healthy family members of aHUS patients[94], in the Exome Variant Server in which exome results of >6500 persons are available (http://evs.gs.washington.edu/EVS/; allele frequency in the European population: 0.6%), and in four non-aHUS patients in our in-house database of 1300 exomes (present in two patients with age-related macular degeneration [AMD], a complement-mediated eye disorder in which this variation has been seen in other AMD patients as well[184-186], but also in two patients with mental retardation). Therefore, in our opinion, this variation cannot be the sole disease-causing aberration in these patients, but rather is a predisposing rare polymorphism. None of the screened patients were positive for αFH.
One patient with a previously described *FI* mutation[157] was taken along as a positive control. Informed consent to study DNA by means of whole exome sequencing was given by all patients or their parents according to the guidelines of the ethical committee of the Radboud university medical centre (CMO 2012/301).

Whole exome sequencing, data analysis and filtering strategy

Genomic DNA was extracted from peripheral blood samples and purified according to the manufracturer's protocol using a Qiagen QIAmp blood maxi kit (Qiagen, Venlo, The Netherlands). DNA purity and degradation was checked on agarose gels. The 50MB Agilent's SureSelect v4 human exome design (Agilent, Santa Clara, CA, USA) was used to capture and enrich the exomes. After

amplification, a SOLiD4 sequencer (Life Technologies, Foster City, CA, USA) was used to sequence the reads.

By means of the SOLiD BioScope software version 1.3, reads were mapped to the hg19 reference genome and they were annotated using an in-house annotation pipeline.[181] Synonymous variants, deep intronic, intergenic, and UTR variants were excluded, as were variants with less than 15% variation and/or less than five variant reads. All variants in genes already associated with aHUS were checked for potential pathogenicity. In all other genes, common variants found in dbSNP or present in >1% in an in-house database of 1300 exomes were filtered. Remaining non-synonymous or splice site variants were prioritized based on a list of possible candidate genes involved in the complement system, coagulation system, and innate immunity, and genes encoding components of endothelial cells and the glomerular basement membrane (Supplementary information). Potentially pathogenic variants were confirmed by Sanger sequencing.

Results and discussion

With the use of exome sequencing we identified on average 48015 variants in our patients. Variants that were present in the dbSNP database or deep intronic variants, true substitutions, variants present in more than 1% of our in-house database with whole exome results of >1300 individuals, and aberrations that had a read percentage of less than 15% were excluded. This resulted on average in 228 sequence variants per patient (Table 5.1). The *CFI* mutation previously detected in patient TMA10 and the associated polymorphism in *C3* were used as a positive control. Both variants were detected using this exome strategy. No other potentially pathogenic sequence variations in any of the other candidate genes involved in the complement system, the coagulation system, or the innate immunity were identified in our aHUS cohort.

The overall horizontal coverage of genes previously associated with aHUS was using a SOLiD sequencer 82.1% (170/207 exons showed a vertical coverage >10 reads), as shown in Table 5.2. Overall coverage of the genes of our candidate list was 80.4% (Supplementary information); in genes with only one exon not present, this was usually exon 1. None of the coding exons of *CFHR1* and *THBD* showed coverage of 10 reads or more throughout the exon; this is also true for short consensus repeats (SCR) 19 and 20 of *CFH* (Figure 5.1). SNPs located in regions of polymorphic deletions of the genes *CFHR1* and *CFHR3*, associated with the presence of αFH[81], do show >10x vertical coverage.

Table 5.1. Number of identified sequence variation with whole exome sequencing on a SOLiD4 sequencer. Variants are prioritized according to a work flow previously published.[181] Common variants present in dbSNP, deep intronic, intergenic, and UTR variants, synonymous variants, variants present in >1% in an in-house database of 1300 exomes and variants with less than 15% variation and/or less than five variant reads were excluded.

	HUS1	HUS2	HUS3	HUS4	HUS5	HUS6	HUS7	HUS8	HUS9	HUS10
Total identified variations	49967	48758	47547	46044	47491	47894	49049	47200	47686	48515
No dbSNP match	2869	2783	2782	2698	2806	2666	2654	2588	2683	2848
Present in exon or canonical splice site	750	735	753	763	776	701	692	690	664	731
False substitution	557	536	527	566	557	522	489	516	497	543
Present in <1% of in-house database	304	271	281	311	306	258	217	240	243	263
% reads >15%	**255**	**242**	**239**	**255**	**262**	**217**	**187**	**197**	**199**	**223**

Lack of coverage in the SCR19 and 20 of *CFH* posses a potential problem if one wants to use WES as the main clinical sequencing approach. More than 100 different mutations have been identified in *CFH* in aHUS patients and >50% of these are located in SCR20.[78] If this part of the gene is not detected using WES, the diagnostic yield of this approach will be low. Furthermore, a number of SNPs of the associated haplotypes in *CFH* and *CD46* are in deep intronic locations (i.e. rs2796267, which is located 547 bases before the first coding nucleotide of *CD46*) and are also not detected simply because these deep intronic targets are missing in the design.

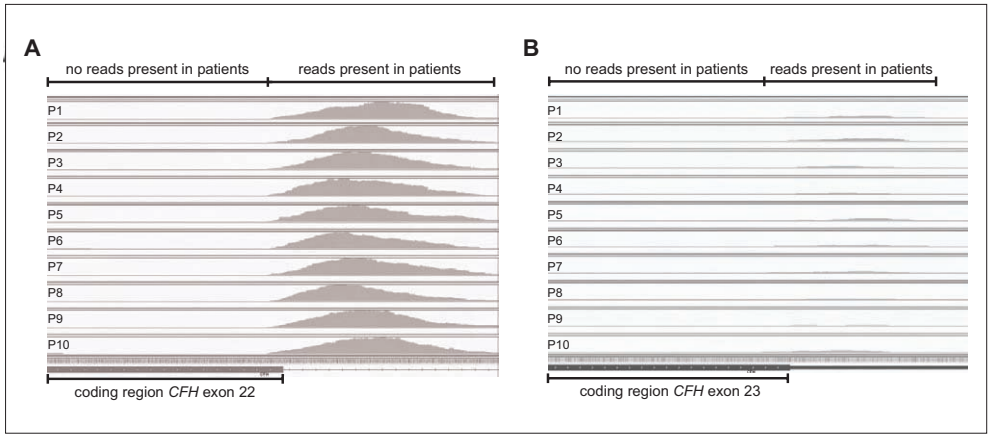

Figure 5.1. Representative visualization of the lack of coverage of exon 22 (A) and exon 23 (B) of *CFH*, the hotspot for mutations in aHUS, after exome sequencing on a SOLiD4 sequencer. The coding regions of the exons and the locations where reads are present in the patients are depicted.

There are several other possible reasons why we might not have identified genetic aberrations in other genes. First, in the last few years, the dbSNP database has grown tremendously due to next generation sequencing. This might impact filter strategies. For instance, the p.Arg1210Cys variant in *CFH*, which clearly influences the binding of FH to C3b[152], nowadays has a dbSNP number (rs121913059) even though it has a frequency in the European population of only 0.03% (2/6501 in the Exome Variant Server, http://evs.gs.washington.edu/EVS/), and based on that would have been excluded from our candidate list. All identified variations in associated genes (Table 5.2), including those with a dbSNP number, have been checked for pathogenicity., Second, it is very well possible we discarded a pathogenic mutation in one of the other candidate genes while prioritizing the identified variants. Third, a disease causing variant outside the coding sequences or the presence of copy number variations (CNVs) can be other reasons why we were unable to identify the genetic cause of aHUS in our patients. This shows the importance to develop robust diagnostic filter strategies and to improve in algorithms to detects clinical relevant variants or CNVs.

Table 5.2. Coverage of genes associated with atypical hemolytic uremic syndrome after whole exome sequencing on a SOLiD4 sequencer and a HiSeq2000. A read depth threshold of 10 reads within the entire exon was used be called covered.

Gene	Number of exons	Percentage covered (>10 reads) on a SOLiD4 sequencer	Percentage covered (>10 reads) on a HiSeq2000
CFH	23	86.9% (20/23)	86.9% (20/23)
CFI	13	100% (13/13)	100% (13/13)
CD46	14	100% (14/14)	100% (14/14)
C3	41	95.1% (39/41)	97.6% (40/41)
CFB	18	100% (18/18)	100% (18/18)
THBD	1	0% (0/1)	100% (1/1)
CFHR1	6	0% (0/6)	66.7% (4/6)
CFHR2	5	80.0% (4/5)	80.0% (4/5)
CFHR3	6	50.0% (3/6)	66.7% (4/6)
CFHR4	10	80.0% (8/10)	80.0% (8/10)
CFHR5	10	90.0% (9/10)	90.0% (9/10)
ADAMTS13	29	79.3% (23/29)	93.1% (27/29)
DGKE	12	81.8% (9/11)	100% (11/11)
PLG	19	52.6% (10/19)	68.4% (13/19)
Overall percentage of exons covered		**82.1% (170/207)**	**89.9% (186/207)**

The described data set was obtained through exome sequencing on a SOLiD4 sequencer and the bioscope software,. In the meantime however, other NGS-sequencing platforms and enrichment kits have been introduced The Illumina HiSeq2000, for instance, is now routinely used for clinical exome sequencing with a minimal median coverage of 75x throughout the exome compared with only ~30

x on a SOLiD4 sequencer. Also, coverage in aHUS candidate genes has increased tremendously using the HiSeq2000 (*THBD* for instance is now completely covered with this approach; Table 5.2), However, exon 23 of *CFH* is still shows lack of complete horizontal coverage (Figure 5.2).

A recent best practice guideline of the Dutch Society for Clinical Genetic Laboratory Diagnostics[187], recommend to use diagnostic yield (the established percentage of molecular diagnoses, also explained as the number of patients that receive a molecular confirmation) as a selection criterion for the performance of NGS based testing. The guideline proposes that a list of 'core disease genes' should be used containing essential/critical genes for a disease, in this case the associated genes of Table 5.2. To be able to implement an NGS-based, the proposed test should result in a diagnostic yield that at least matches that of Sanger sequencing, with the same sensitivity and specificity. As exon 23 of *CFH* is not covered with WES, even with newer techniques than used for this manuscript, you miss the disease-causing mutation in up to 7.5% of the patients.[78] Thus, if we follow this guideline the sole application of WES alone will lead to a lower diagnostic yield and implementation of WES as a sequencing strategy is not justified yet. The only way to overcome this issue is to use Sanger sequencing to complete mutation analysis of the essential missing coding sequences.

As an alternative NGS approach, one could design a targeted analysis of the 207 exons of the core disease gene list using a benchtop sequencer, i.e. the iontorrent PGM (Thermofisher) or the MiSeq (Illumina), Sanger sequencing might still be needed to cover exons in regions that are difficult to sequence by NGS, but the overall time to results is likely much faster when compared to WES. Also the capacity on these benchtop sequencer might be a more cost effective way to get to diagnostic valid results. Whole genome sequencing, although an option in the future as well, is in terms of efficiency and costs not yet an attractive alternative to exome sequencing approaches.

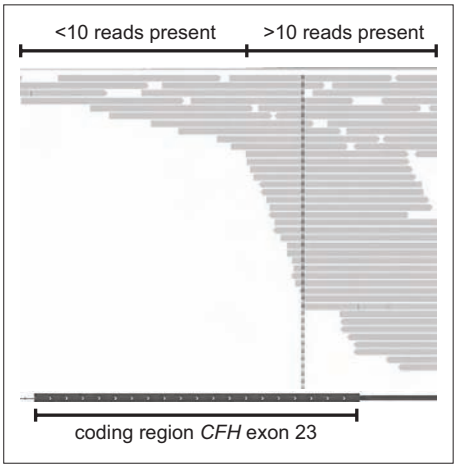

Figure 5.2. Visualization of the lack of coverage of exon 23 of *CFH* after exome sequencing on an Illumina HiSeq2000. The coding region of the exon and the locations within the exon with a read depth threshold of 10 are depicted.

For now, we propose a diagnostic routing consisting of a combination of amplicon based (Sanger) sequencing of the *CFH* region (at least SCR20 and the complete coding region of *CFHR1-5*) and the deep intronic SNPs of the associated haplotypes, in combination with a NGS based gene panel targeting the associated aHUS genes. This strategy will likely match the diagnostic yield to at least that of Sanger sequencing only, but it will be achieved in a time and cost effective manner.

Acknowledgements

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Supplementary information.

List of candidate genes encoding proteins of the complement system, coagulation system, and innate immunity, endothelial cell components, and glomerular basement membrane components. For each gene, the percentage of covered exons for P1 on a SOLiD4 sequencer is shown. A vertical coverage of >10 reads over the entire exon was used to call an exon completely covered. Genes of which exon 1 was not present, are indicated with an asterisk.

Name	Coverage	Name	Coverage	Name	Coverage	Name	Coverage
ACAN	55.6% (10/18)*	*CD74*	100.0% (6/6)	*FLT4*	93.3% (28/30)*	*ITGB5*	80.0% (12/15)*
ACE	72.0% (18/25)*	*CD79A*	80.0% (4/5)	*FN1*	78.2% (36/46)*	*ITGB6*	80.0% (12/15)
ADAMTS2	90.1% (20/22)*	*CD79B*	100.0% (6/6)	*GP1Ba*	50.0% (1/2)*	*ITGB7*	87.5% (14/16)*
ADAMTS13	79.3% (23/29)	*CD93*	50.0% (1/2)	*GP6*	87.5% (7/8)	*ITGB8*	78.6% (11/14)
ADAMTS17	86.4% (19/22)*	*CD97*	72.2% (13/18)	*GPC1*	77.8% (7/9)*	*KDR*	96.7% (29/30)*
ADAMTS19	95.7% (22/23)	*CFB*	100.0% (18/18)	*GPC2*	50.0% (5/10)*	*LY96*	80.0% (4/5)
ADAMTS20	94.9% (37/39)	*CFD*	20.0% (1/5)*	*GPC3*	77.8% (7/9)*	*MASP1*	96.8% (15/16)
AGT	100.0% (4/4)	*CFH*	86.9% (20/23)	*GPC4*	88.9% (8/9)	*MASP2*	72.7% (8/11)*
AGTR1	50.0% (1/2)*	*CFHR1*	0.0% (0/6)*	*GPC5*	75.0% (6/8)*	*MBL2*	75.0% (3/4)*
APOB	93.1% (27/29)*	*CFHR2*	80.0% (4/5)	*GPC6*	77.8% (7/9)	*MIF*	66.7% (2/3)
BDKRB2	66.7% (2/3)	*CFHR3*	50.0% (3/6)	*HSPG2*	79.4% (77/79)*	*MMACHC*	75.0% (3/4)
C1QA	50.0% (1/2)*	*CFHR4*	80.0% (8/10)	*ICAM1*	57.1% (4/7)*	*PDGFA*	42.9% (3/7)*
C1QB	50.0% (1/2)*	*CFHR5*	90.0% (9/10)	*ICAM2*	100.0% (4/4)	*PDGFB*	66.7% (4/6)
C1QBP	66.7% (4/6)*	*CFI*	100.0% (13/13)	*ICAM3*	71.4% (5/7)	*PDGFRA*	95.7% (22/23)
C1QC	33.3% (1/3)*	*CFP*	30.0% (3/10)*	*ICAM4*	66.7% (2/3)*	*PDGFRB*	73.9% (17/23)*
C1QL1	50.0% (1/2)*	*CLU*	77.8% (7/9)*	*IFNG*	75.0% (3/4)*	*PGF*	50.0% (3/6)*
C1QL2	50.0% (1/2)*	*CPB2*	90.9% (10/11)	*IL1A*	100.0% (7/7)	*PLAT*	92.3% (12/13)
C1QL3	50.0% (1/2)*	*CR1*	42.6% (20/47)*	*IL2*	100.0% (4/4)	*PLG*	52.6% (10/19)*
C1QL4	0.0% (0/2)*	*CR1L*	50.0% (6/12)	*IL6*	100.0% (5/5)	*PROC*	44.4% (4/9)*
C1R	100% (9/9)	*CR2*	94.7% (18/19)*	*IL8*	100.0% (4/4)	*PROCR*	75.0% (3/4)
C1RL	83.3% (5/6)	*DGKE*	81.8% (9/11)*	*IL10*	80.0% (4/5)	*PROS1*	46.7% (7/15)
C1S	91.7% (11/12)	*F2*	85.7% (12/14)	*IL12A*	85.7% (6/7)*	*PROZ*	87.5% (7/8)
C2	92.9% (13/14)	*F2R*	50.0% (1/2)*	*IL12RB1*	81.3% (13/16)	*SDC2*	100.0% (5/5)
C3	95.1% (39/41)	*F3*	83.3% (5/6)	*ITGA1*	100.0% (29/29)	*SERPINB2*	100.0% (8/8)
C3aR1	100.0% (1/1)	*F5*	92.0% (23/25)	*ITGA2*	86.7% (26/29)	*SERPINC1*	100.0% (7/7)
C4A	0.0% (0/41)*	*F7*	88.9% (8/9)	*ITGA2B*	76.7% (23/30)	*SERPINE1*	77.8% (7/9)*
C4B	0.0% (0/41)*	*F8*	80.8% (21/26)*	*ITGA3*	69.2% (18/26)*	*SERPING1*	87.5% (7/8)*
C4BPA	75.0% (9/12)	*F9*	100.0% (8/8)	*ITGA4*	89.2% (25/28)	*TGFB1*	57.1% (4/7)*
C4BPB	100.0% (7/7)	*F10*	91.7% (11/12)	*ITGA5*	86.7% (26/30)*	*THBD*	0.0% (0/1)*
C5	90.2% (37/41)	*F11*	83.3% (10/12)	*ITGA6*	84.0% (21/25)	*TLR1*	0.0% (0/4)*
C5aR1	100.0% (2/2)	*F12*	57.1% (8/14)	*ITGA7*	72.0% (18/25)*	*TLR2*	100.0% (3/3)
C6	88.9% (16/18)	*F13A1*	92.9% (13/14)*	*ITGA8*	86.7% (26/30)*	*TLR3*	100.0% (5/5)
C7	94.4 (17/18)	*F13B*	91.7% (11/12)	*ITGA9*	82.1% (23/28)*	*TLR4*	100.0% (3/3)
C8A	100.0% (11/11)	*FCN1*	100.0% (9/9)	*ITGA10*	93.3% (28/30)	*TLR5*	100.0% (6/6)
C8B	92.3% (12/13)	*FCN2*	100.0% (7/7)	*ITGA11*	76.7% (27/30)*	*TLR6*	100.0% (2/2)
C8G	85.7% (6/7)	*FCN3*	100.0% (8/8)	*ITGAD*	86.7% (26/30)	*TLR7*	100.0% (3/3)
C9	100.0% (11/11)	*FGA*	83.3% (5/6)	*ITGAE*	87.1% (27/31)	*TLR8*	100.0% (2/2)
CD14	100.0% (2/2)	*FGB*	87.5% (7/8)	*ITGAL*	71.0% (22/31)	*TNF*	75.0% (3/4)
CD19	78.6% (11/14)	*FGD5*	100.0% (20/20)	*ITGAM*	80.0% (24/30)*	*VEGFA*	87.5% (7/8)*
CD36	92.8% (13/14)	*FGG*	100.0% (10/10)	*ITGAV*	85.7% (24/28)	*VEGFB*	85.7% (6/7)*
CD40	100.0% (9/9)	*FGL1*	100.0% (7/7)	*ITGAX*	86.7% (26/30)	*VEGFC*	71.4% (5/7)*
CD44	90.0% (9/10)*	*FGL2*	100.0% (2/2)	*ITGB1*	75.0% (12/16)	*VTN*	62.5% (5/8)
CD46	100.0% (14/14)	*FGR*	92.3% (12/13)	*ITGB2*	75.0% (12/16)	*VWF*	55.8% (29/52)
CD55	81.9% (9/11)*	*FIGF*	100.0% (15/15)	*ITGB3*	87.5% (14/16)*		
CD59	75.0% (3/4)*	*FLT1*	96.7% (29/30)*	*ITGB4*	90.0% (36/40)	**TOTAL**	**80.4% (1995/2480)**

Chapter 6

Atypical hemolytic uremic syndrome in children: complement mutations and clinical characteristics

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Abstract

Mutations in complement Factor H (*CFH*), factor I (*CFI*), factor B (*CFB*), thrombomodulin (*THBD*), complement component 3 (*C3*), and membrane cofactor protein (*CD46*), and autoantibodies against factor H (αFH) with or without a homozygous deletion in FH-related protein 1 and 3 (Δ*CFHR1/3*) predispose development of atypical hemolytic uremic syndrome (aHUS). Different mutations in genes encoding complement proteins in 45 pediatric aHUS patients were retrospectively linked with clinical features, treatment, and outcome. In 47% of the study participants, potentially pathogenic genetic anomalies were found (5x *CFH*, 4x *CD46*, 4x *C3*, 3x *CFI*, 2x *FB*, and 6x αFH, of which five had Δ*CFHR1/3*); four patients carried combined genetic defects or a mutation together with αFH. In the majority (87%), disease onset was preceded by a triggering event; in 25% of cases diarrhea was the presenting symptom. More than 50% had normal serum C3 levels at presentation. Relapses were seen in half of the patients, and there was renal graft failure in all except one case following transplant. Performing adequate DNA analysis is essential for treatment and positive outcome in children with aHUS. The impact of intensive initial therapy and renal replacement therapy, as well as the high risk of recurrences of aHUS in renal transplant, warrants further understanding of the pathogenesis, which will lead to better treatment options.

Introduction

Atypical hemolytic uremic syndrome (aHUS) is a rare disease characterized by hemolytic anemia, thrombocytopenia, and acute renal failure secondary to thrombotic microangiopathy.[36] It is distinguished from typical or Shigatoxin-producing *Escherichia coli* (STEC) HUS by the absence of STEC infection. In recent years, aHUS had been found to be associated with complement alternative pathway dysregulation. In almost 60% of aHUS patients, mutations in genes encoding complement-regulation proteins are reported.[29, 139] Aside from mutations in these genes, aHUS has also been reported in cobalamin C (cblC) disease caused by mutation in the cblC *MMACHC* gene.[188]

With respect to complement-regulation proteins, most mutations are seen in factor H (protein: FH; gene: *CFH*), but mutations in membrane cofactor protein (*MCP* or *CD46*) and factor I (FI; *CFI*) are also known to predispose development of aHUS.[3, 5, 6, 9-11] Additionally, gain-of-function mutations in factor B (FB; *CFB*) and complement C3 (*C3*) are reported.[74, 75, 90, 189] Some patients have combined mutations. Functional FH deficiency due to autoantibodies against FH (αFH) have recently been described, which is highly associated with a polymorphic homozygous deletion of genes encoding factor H-related protein 1 and 3 (Δ*CFHR1/3*).[81, 82, 137, 141] Finally, mutations in the thrombomodulin genen (*THBD*), encoding membrane-bound thrombomodulin that modulates complement activation on cell surfaces, have been reported in aHUS.[73] Genetic complement anomalies are thought to be risk factors rather than the only cause of the disease, as patient relatives with the same mutation do not always exhibit disease activity. Atypical HUS represents approximately 10% of pediatric HUS cases. More than 80% of cases are sporadic, with a better prognosis overall than familial cases, which have a higher mutation rate.[29, 90] Knowledge about the impact of mutations in complement-regulating genes upon disease characteristics, response to treatment, and outcome in pediatric aHUS patients could help define therapeutic guidelines and be of prognostic value. Therefore, the aim of this study was to: (1) document the frequency of known genetic complement abnormalities in a cohort of Dutch and Belgian children diagnosed with aHUS, and (2) describe and compare disease characteristics, treatment, and clinical outcome of these children.

Methods

Study population

From 2001 to 2010, genomic DNA was analyzed for genetic disorders in complement (regulating) genes (*CFH*, *CFI*, *CD46*, *CFB*, *C3*, *THBD*, and Δ*CFHR1/3* with αFH) in 45 Dutch and Belgian pediatric

patients diagnosed with aHUS by pediatric nephrologists at six university medical centers in The Netherlands and one in Belgium. Diagnosis of aHUS was based on the following findings: hemolytic anemia characterized by a hemoglobin (Hb) level <10 g/dl, thrombocytopenia with a platelet count <150x10^9/l, elevated levels of lactate dehydrogenase (LDH), and a negative Coombs test, all in association with acute renal impairment. Patients with STEC HUS were excluded by stool cultures, polymerase chain reaction (PCR), and/or serologic antibodies against LPS O157. Genetic analysis were combined with retrospectively obtained information about patient clinical presentation, received treatment, and outcome. Genetic analysis of 25 of these 45 patients has been published before in a cohort of adults and children by Westra *et al.*[72] Patients in that and this study are indicated in the authors' earlier study by the following numbers: 2, 3, 4, 5, 6, 9, 15,16, 18, 21, 24, 33, 36, 39, 40, 41, 42, 43, 44, 45, 48, 54, 58, 62. When two or more family members of the same family were affected by the disease at least six months apart, it was considered as familial aHUS. Sporadic aHUS patients had no family history of the disease. Complete remission was defined by normalization of hematologic parameters (Hb >10 g/dl, thrombocytes >150x10^9/l, LDH <450 U/l) and renal function (glomerular filtration rate [GFR] >80 ml/min/1.73 m^2, no proteinuria). In patients with partial remission, hematologic normalization was seen, but with renal sequelae (GFR <80 ml/min/1.73 m^2 and/or proteinuria and/or hypertension). The presence of proteinuria was defined as >0.2 g/24 h or positive dipstick for proteins. Hypertension was defined as a consistent blood pressure >p95 for age and length. Recurrence was reported when a new episode of aHUS presented >4 weeks after remission. Chronic treatment was defined by the use of dialysis or plasma therapy for >3 months. The average follow-up time was 7.5 years, varying from two weeks in one patient previously diagnosed and still hospitalized at time of inclusion, to 17 years in one patient who reached adulthood. Permission to study DNA material was given by all patients an/or parents.

Genetic analysis of genes encoding FH*,* FI*,* CD46*,* C3*,* FB*, and* THBD

Genomic DNA was amplified for *CFH* [National Centre for Biotechnology Information (NCBI) RefSeq NM_000186.3], *CFI* (NM_000204.3), *CD46* (NM_002389.3), *C3* (NM_000064.2), *CFB* (NM_001710.4), and *THBD* (NM_000361.2) by means of PCR. Primer data are available upon request. Fragments included DNA sequences of the individual exons and the splice donor and acceptor site. Amplimers were subjected to double-stranded DNA sequence analysis on an ABI 3130 *xl* Genetic Analyzer (Applied Biosystems). Sequence analysis was performed using Sequencher 4.8 software. Detected genetic aberrations were confirmed on a second PCR product. Genomic DNA from >100 healthy ethnically matched control individuals was used to confirm sequence variations that might be

potentially pathogenic. Potential pathogenicity of genetic alterations was further checked in the literature, evolutionary conservation, and *in silico* prediction programs (Sorting Intolerant From Tolerant [SIFT; http://sift.jcvi.org/] and Polymorphism Phenotyping v2 [PolyPhen-2; http://genetics.bwh.harvard.edu/pph2/]). For evolutionary conservation, the 17-way vertebrate alignment from the University of Califormia Santa Cruz (UCSC) Genome Browser (http://genome.uscs.edu) was used.

Autoantibodies against factor H (αFH) and homozygous deletion of FHR-1 *and* FHR-3 *genes*

Serum samples were tested for the presence of αFH by means of enzyme-linked immunnosorbent assay (ELISA), as described previously.[81] A positive control sample was kindly obtained via Dr. Dragon-Durey (Paris, France). Test results were considered positive if they were above twice the standard deviation calculated from controls, and samples were tested at least three times independently, as described by Dragon-Durey *et al*.[137] A homozygous deletion of *CFHR1* and *CFHR3* was identified as described previously.[72] In short, genomic DNA was amplified by PCR using specific primers located in a 100-kb region downstream of *CFH*. Amplification of one of the fragments failed in case of Δ*CFHR1/3*. This method does not detect a heterozygous deletion of the two genes or the presence of a hybrid *CFH/CFHR1* gene.

Results

Patients

Data on *CFH*, C*FI*, *CD46*, *CFB*, and *C3* mutations characteristics are reported in Table 6.1. Data on patient characteristics at presentation, treatment, and clinical outcome for each type of mutation are reported in Table 6.2, Table 6.3, and Table 6.4. Due to the small number of patients per mutated complement protein, data were analyzed descriptively without statistical comparisons. Clinical data on the four patients with a combined mutation or a mutation in combination with a deletion in *CFHR1/3* and αFH are given in Table 6.5. Forty-five patients diagnosed with aHUS from six different university medical centers in The Netherlands and one in Belgium were included. Approximately as many males as females were included in this study.

Genetic anomalies

In 20 out of 45 patients (44%) heterozygous mutations in complement regulating genes and/or autoantibodies against FH were found, all but one with a homozygous deletion in *CFHR1/3* (Table

6.1). Five patients (overall 11%) carried a mutation in *CFH*, four (overall 9%) in *CD46*, and also four in *C3* (overall 9%). Three patients (overall 7%) had a mutation in *CFI* and two (overall 4%) in *CFB*. Six patient (overall 13%) had autoantibodies against FH. Two of these patients with a genetic anomaly carried a combined mutation (*CFI*/*CD46* and *CFI/C3*). One patient had a *CFI* mutation and αFH with Δ*CFHR1/3*, and in one patient αFH were seen in combination with a *C3* mutation but without Δ*CFHR1/3*. Patients with combined mutations are included in both their mutation groups in Table 6.2, Table 6.3, and Table 6.4. Characteristics at onset, treatment, and outcome of the four patients with combined genetic anomalies are also summarized separately (Table 6.5). No mutations in *THBD* were found in our cohort.

Clinical characteristics

Familial vs sporadic

Eleven patients (24%) had familial aHUS, of whom all except two had an associated mutation. We included one family with three sisters with a *CFH* mutation and another family with two cousins with a mutation in *CFB*. Of the other six patients with familial aHUS, only one family member was

Table 6.1. Characteristics of mutations found in *CFH*, *CFI*, *CD46*, *CFB*, and *C3* genes in 17 of 45 pediatric patients with atypical hemolytic uremic syndrome (aHUS).

Subject code, exon/intron	Mutation	Effect	Known/unknown[a]
Complement factor H			
Exon 23 (n = 1)	c.3628C > T	p.Arg1210Cys	Known
Exon 23 (n = 3)	c.3572C > T	p.Ser1191Leu	Known
Exon 9 (n = 1)	c.1198C > A	p.Gln400Lys	Known
Complement factor I			
Intron 12 (n = 1[b])	g.IVS12 + 5G > T	Splice score decrease from 0.93 to 0.86	Known
Exon 9 (n = 1[c])	c.1019T > C	p.Ile340Thr	Known
Exon 10 (n = 1[c])	c.1071T > G	p.Ile357Met	Unknown
Membrane cofactor protein			
Exon 6 (n = 1[b])	c.811-816delGACAGT	p.delAsp271-Ser272	Known
Complement C3			
Exon 4 (n = 4[d])	c.481C > T	p.Arg161Trp	Known [175]
Complement factor B			
Exon 18 (n = 2)	c.967A > G	p.Lys323Glu	Known

Every single mutation is mentioned separately. Patients without mutations but with a homozygous deletion in Δ*CFHR1/3* and/or autoantibodies against factor H are not listed in this table. Of three patients with an *CD46* mutation, DNA analysis was not performed in our laboratory. Mutation and effect are therefore not listed above.

[a] According to www.fh-hus.com and [175].

[b] Patient carrying both *CFI* and *CD46* mutations.

[c] One patient carrying both *CFI* and *C3* mutations.

[d] Patient carrying *CFI* mutation and a homozygous deletion in *CFHR1/3* and autoantibodies against FH (αFH).

included in the study because further data were not available. All four patients in this study with a *C3* mutation have familial aHUS. Sporadic aHUS was seen in all four patients with a *CD46* mutation and all five patients with αFH and Δ*CFHR1/3*.

Onset

In ten patients (22%), aHUS onset was before 1 year of age, with the youngest patient being only 1 month old. In only one of these patients was a mutation found (*C3*/αFH), and only two patients had familial aHUS. The majority of aHUS patients (53%) presented between the age of 1 and 7 years, which is also the most common age for children with typical HUS to present with the disease, which suggests that age at onset cannot always help distinguish between typical and aHUS. In 38/45 patients, aHUS onset followed a triggering event, such as gastrointestinal symptoms (74%), upper respiratory tract infection (45%), and fever (32%). A combination of triggering events was seen as well; for instance, vomiting and fever were seen in 8/45 patients. Diarrhea was seen in 11 patients, five of whom also had fever. One patient developed aHUS a few days after a hepatitis B vaccination, and one patient was positively tested for a *Bordetella pertussis* infection a few days before aHUS onset. Other triggers were infection with *Haemophilus influenza* (n=1) and a streptococcal infection (n=1).

Hypertension (blood pressure >95 percentile for age) was present at first presentation in 71% of patients. Six patients had involvement of the central nervous system due to malignant hypertension or cerebral infarction during their first aHUS flare. Two of these patients had a mutation (*C3* and *CFB*) and one patient had αFH with Δ*CFHR1/3*. These three patients had severe seizures; the other three patients were extremely agitated of had reduced consciousness. One patient had additional visual handicap, and one showed apraxia and aphasia. Pancreas involvement during the first episode was seen in only two patients, both without a genetic defect. In 18 of 37 patients (49%) for whom data were available, biochemical evaluation showed reduced C3 levels (minimum 210 mg/l, reference range 900-1800 mg/l). Four of these patients had a mutation (2x *CFB*, 1x *CFI*, 1x *C3*), and four had αFH, of which three also had Δ*CFHR1/3*. Low C3 levels were always linked with high levels of the activation product C3d. However, we also found elevated levels of C3d in six patients with normal levels of C3. Of the 25 genetically undefined patients, 11 showed reduced C3 levels, and in seven, elevated levels of C3d were found, showing evidence of alternative pathway activation. Remarkably, reduced C4 levels were seen in six of the 31 tested patients (19%), with an extreme lowest level of 30 mg/l (reference range 150-400 mg/l). A systemic disease, such as systemic lupus erythamatosis (SLE) of cryoglobinemia as cause of these low C4 levels was ruled out because of the

clinical and laboratory results combined with medical history. One of the patients had a *FH* mutation.

Treatment and outcome of first episode

The majority of patients (60%) were treated with plasma therapy, including plasma infusion (PI) and/or plasma exchange (PE) (Table 6.2). Plasma therapy was applied in 12 patients with a genetic defect vs 15 patients without a known genetic anomaly. In 11 patients, plasma therapy was combined with dialysis. Nine patients were treated conservatively, three of whom had a genetic defect (1x *C3*, 1x *CD46*, 1x αFH with Δ*CFHR1/3*). In only 3/20 patients (15%) with a genetic defect (2x *CFH*, 1x *CD46*) was complete remission reached after the first episode, compared with 9/24 patients (38%) in the other group. One of these patients (*CFH* mutation) was treated with plasma therapy (four infusions with fresh frozen plasma), one with dialysis (*CFH* mutation), and one patient recovered spontaneously (mutation in *CD46*). All other patients with a genetic anomaly reached partial remission after the first flare. Of the nine patients without plasma therapy or dialysis, only three reached complete remission (1x *CD46*, 2x no genetic anomaly).

Chronic plasma therapy and/or dialysis was applied more often in the group with than in the group without a genetic defect (40% vs 30%). One patient died during the first aHUS episode due to consequences of the disease. This was the youngest patient in the study, only 1 month old. A genetic defect in complement proteins was not found in this patient.

Long-term outcome

Almost half of the aHUS patients (n = 21) had a relapse (Table 6.3). In the group with a genetic anomaly, 13/20 (65%) of patients relapsed. In the group without known anomalies, relapse occurred in only 8/25 (32%) patients. A total of 43 relapses was seen, of which 29 (67%) occurred in patients with any of the genetic subtypes. A maximum of five relapses was seen in one patient with αFH and Δ*CFHR1/3*. Of all patients with a relapse, 38% had familial aHUS. Most relapses occurred after a viral infection, usually of the upper respiratory tract. Time between aHUS onset and the first relapse varied widely, from 1 month after remission to a maximum of 8.5 years. In 9/21 patients, aHUS relapsed within 1 year, five had a genetic defect (1x *CFH*, 1x *CD46*, 1x *C3/CFI*, and 2x αFH with Δ*CFHR1/3*). Ten patients with a relapse were initially treated with plasma therapy, eight of whom received chronic plasma therapy. In most patients, plasma therapy was intensified during the relapse.

Table 6.2. Characteristics at onset of atypical hemolytic uremic syndrome (aHUS) in 45 pediatric patients with mutations in *CFH*, *CFI*, *CD46*, *CFB*, and *C3*, with a homozygous deletion in *CFHR1/3* (Δ*CFHR1/3*) and autoantibodies against FH (αFH), as well as in patients without known genetic anomalies.

Parameter	All patients	No genetic defect	Genetic defect	*CFH*	*CFI*	*CD46*	*CFB*	*C3*	*ΔCFHR1/3*	αFH
Male/female	22/23 (45)	12/13 (25)	10/10 (20)	2/3 (5)	3/0 (3)	3/1 (4)	0/2 (2)	2/2 (4)	3/2 (5)	3/3 (6)
Familial/sporadic	11/34 (45)	2/23 (25)	9/11 (20)	3/2 (5)	1/ 2 (3)	0/4 (4)	2/0 (2)	4/0 (4)	0/5 (5)	1/5 (6)
Age of onset										
0-1 year	10 (45)	9 (25)	1 (20)	0 (5)	0 (3)	0 (4)	0 (2)	1 (4)	0 (5)	1 (6)
1-7 years	24 (45)	10 (25)	14 (20)	4 (5)	2 (3)	2 (4)	1 (2)	2 (4)	5 (5)	5 (6)
≥7 years	11 (45)	6 (25)	5 (20)	1 (5)	1 (3)	2 (4)	1 (2)	1 (4)	0 (5)	0 (6)
Symptoms										
Fever	13 (41)	6 (22)	7 (20)	0 (4)	1 (3)	1 (4)	0 (2)	4 (4)	2 (5)	3 (6)
URTI	14 (31)	5 (14)	9 (19)	2 (5)	2 (3)	1 (2)	2 (2)	3 (4)	1 (3)	2 (4)
Oligo/anuria	28 (40)	15 (21)	13 (19)	5 (5)	1 (3)	2 (3)	1 (2)	3 (4)	2 (5)	3 (6)
Gastrointestinal[a]	32 (43)	17 (23)	15 (20)	3 (5)	2 (3)	2 (4)	1 (2)	4 (4)	4 (5)	5 (6)
Hypertension	32 (45)	20 (25)	12 (20)	3 (5)	1 (3)	1 (4)	1 (2)	2 (4)	5 (5)	6 (6)
Biochemical evaluation (mean levels)										
Hemoglobin (g/dl)	7,6 (45)	7,7 (25)	7,5 (20)	7,0 (5)	12,4 (3)	13,7 (4)	12,2 (2)	10,6 (4)	12,7 (5)	12,2 (6)
Platelet count (×10^9/l)	81 (45)	86 (25)	76 (20)	69 (5)	136 (3)	29 (4)	175[b] (2)	114 (4)	51 (5)	50 (6)
LDH (U/l)	3784 (42)	4382 (23)	3062 (19)	1338 (5)	4639 (3)	3688 (3)	1407 (2)	3195 (3)	5147 (5)	4886 (6)
Uremia (mmol/l)	28 (45)	31 (25)	25,6 (20)	27 (5)	28.5 (3)	19,8 (4)	46,6 (2)	19,4 (4)	35,1 (5)	31,5 (6)
Creatinine (mmol/l)	248 (45)	290 (25)	195 (20)	193 (5)	155 (3)	124 (4)	459 (2)	100 (4)	225 (5)	199 (6)
C3 (mg/l)[c]	881 (37)	862 (22)	908 (15)	1276 (5)	805 (2)	933 (3)	335 (2)	810 (3)	717 (3)	705 (4)
C4 (mg/l)[d]	232 (31)	227 (19)	240 (12)	220 (3)	193 (2)	185 (1)	378 (2)	230 (1)	227 (3)	227 (3)

The numbers of patients for whom data were available are reported in parentheses. URTI indicates: upper respiratory tract infection; LDH: lactate dehydrogenase.

[a] Vomiting and/or diarrhea.

[b] Although one criterion of having aHUS is to have serum platelet count <150 × 10^9/l, one patient with thrombocytes of 207 × 10^9/l, familial aHUS, and a *CFB* mutation was included as an exception because of her clear presentation. She was probably diagnosed in an early stage because of her familial background.

[c] Reference range 900–1,800 mg/l.

[d] Reference range 150–400 mg/l.

Table 6.3. Treatment in 45 pediatric atypical hemolytic uremic syndrome (aHUS) patients with mutations in *CFH*, *CFI*, *CD46*, *CFB*, and *C3*, with a homozygous deletion in *CFHR1/3* (Δ*CFHR1/3*) and autoantibodies against FH (αFH), as well as in patients without known genetic anomalies, and the outcomes of their first aHUS episode.

Parameter	All patients	No genetic defect	Genetic defect	*CFH*	*CFI*	*CD46*	*CFB*	*C3*	*ΔCFHR1/3*	αFH
Treatment										
Plasma therapy[a]	27 (45)	15 (25)	12 (20)	3 (5)	2 (3)	3 (4)	2 (2)	1 (4)	3 (5)	4 (6)
Dialysis	20 (45)	12 (25)	8 (20)	2 (5)	1 (3)	2 (4)	1 (2)	2 (4)	1 (5)	1 (6)
PT and dialysis	11 (45)	8 (25)	3 (20)	0 (5)	0 (3)	2 (4)	1 (2)	0 (4)	0 (5)	0 (6)
No PT or dialysis	9 (45)	6 (25)	3 (20)	2 (5)	0 (3)	1 (4)	0 (2)	1 (4)	1 (5)	1 (6)
Outcome										
Complete remission	12 (45)	9 (25)	3 (20)	2 (5)	0 (3)	1 (4)	0 (2)	0 (4)	0 (5)	0 (6)
Partial remission	31 (45)	14 (25)	17 (20)	3 (5)	3 (3)	3 (4)	2 (2)	4 (4)	5 (5)	5 (6)
Chronic PT	12 (44)	6 (25)	6 (20)	2 (5)	1 (3)	1 (4)	0 (2)	0 (4)	3 (5)	3 (6)
Chronic dialysis	7 (45)	3 (25)	4 (20)	2 (5)	1 (3)	0 (4)	1 (2)	1 (4)	0 (5)	0 (6)
Death	1 (45)	1 (25)	0 (20)	0 (5)	0 (3)	0 (4)	0 (2)	0 (4)	0 (5)	0 (6)

The numbers of patients for whom data were available are reported in parentheses.

[a] Plasma infusion and/or exchange

Table 6.4. Long-term patient and renal graft outcomes in 45 pediatric atypical hemolytic uremic syndrome (aHUS) patients with mutations in *CFH*, *CFI*, *CD46*, *CFB*, and *C3*, with a homozygous deletion in *CFHR1/3* (Δ*CFHR1/3*) and autoantibodies against FH (αFH), as well as in patients without known genetic anomalies.

Parameter	All patients	No genetic defect	Genetic defect	*CFH*	*CFI*	*CD46*	*CFB*	*C3*	*ΔCFHR1/3*	αFH
Relapses	21 (43)	9 (23)	12 (20)	3 (5)	1 (3)	2 (4)	1 (2)	3 (4)	3 (5)	3 (6)
No. of relapses	43 (21)	14 (9)	29 (20)	8 (3)	4 (1)	3 (2)	1 (1)	8 (3)	9 (3)	9 (3)
Hypertension	23 (43)	12 (23)	11 (19)	2 (4)	1 (3)	2 (4)	2 (2)	4 (4)	3 (5)	4 (6)
Proteinuria	18 (38)	9 (21)	9 (17)	0 (2)	1 (3)	1 (2)	2 (2)	2 (4)	2 (5)	2 (6)
Renal Tx	7 (44)	3 (24)	4 (20)	2 (5)	1 (3)	1 (4)	0 (2)	1 (4)	0 (5)	0 (6)
No. of Tx	13 (7)	5 (3)	8 (4)	4 (2)	3 (1)	1 (1)	0(0)	3 (1)	0 (0)	0 (0)
No. of relapses in renal graft	10 (13)	4 (5)	6 (8)	5 (4)	2 (3)	0 (1)	0 (0)	2 (3)	0 (0)	0 (0)
Death	4 (45)	2 (25)	2 (20)	0 (5)	0 (3)	1 (4)	0 (2)	0 (4)	1 (5)	1 (6)

The numbers of patients for whom data were available are reported in parentheses. 'Tx' indicates transplantation(s).

In 54% of patients, chronic hypertension was seen after the first flare; in 47%, proteinuria sustained. Most of these patients had to be treated for several months with antihypertensive agents. In four patients, neurological involvement was seen later during the course of disease. Two of these patients presented with seizures due to malignant hypertension (*CD46* and *C3/CFI*); one patient without a genetic defect had reduced consciousness (computed tomography [CT] scan showed no abnormalities), and one patient had dysphasia and sensibility disorders due to an arteria cerebralis media stenosis (*CFH*). Another patient developed necrotizing pancreatitis with transient diabetes mellitus 10 years after first presentation, for which a partial pancreatectomy was performed. No mutation or deletion was found in this patient.

Seven patients (2x *CFH*, 1x *CD46*, 1x *C3/CFI*, and three without a known genetic defect) received a total number of 13 renal transplants, but in 10/13 grafts, aHUS recurred. Three of these patients had familial aHUS. No recurrence was seen in patient with *CD46* mutation. Time between

Table 6.5. Characteristics at disease onset, during treatment, and outcome of four pediatric atypical hemolytic uremic syndrome (aHUS) patients with combined mutations and/or with a homozygous deletion in *CFHR1/3* (Δ*CFHR1/3*) and/or autoantibodies against FH (αFH).

Parameter	Patient 1	Patient 2	Patient 3	Patient 4
Combined mutation	*CFI/CD46*	*CFI/C3*	*CFI/CFHR1/3*/αFH	*C3*/αFH
Gender	Male	Male	Male	Female
Age at onset (years)	13 10/12	1 2/12	6 6/12	6/12
Hemoglobin (g/dl)	7.2	6.3	9.7	5.5
Platelet count (×10^9/l)	13	276	120	44
Creatinine (mmol/l)	94	76	294	72
C3 (mg/l)[a]	1000	–	610	670
C4 (mg/l)[b]	185	–	200	–
Plasmatherapy	yes	No	yes	Yes
Dialysis	No	Yes	No	No
Complete remission	No	No	No	No
Partial remission	Yes	Yes	Yes	Yes
Chronic plasmatherapy	No	No	Yes	No
Chronic dialysis	No	Yes	No	No
Death	No	No	No	No
Relapse	No	Yes	No	No
No. of relapses	–	4	–	–
Renal transplant	No	Yes	No	No
No. of renal transplants	–	3	–	–
No. of relapses in grafts	–	2	–	–
Death	No	No	No	No

The numbers of patients for whom data were available are reported in parentheses.

[a] Reference range 900–1,800 mg/l.

[b] Reference range 150–400 mg/l.

transplantation and first relapse in renal graft patients varied between six days and four years. Additionally, one graft was lost due to arteria renalis thrombosis one day after transplantation; one graft was lost due to rejection after four years. One patient died during the acute phase of aHUS. Three other patients died later during the course of disease. One patient with no genetic defect died nine years after onset because of massive cerebral bleeding due to malignant hypertension. Another patient with an *CD46* mutation, who had no recurrence of aHUS in the renal transplant, developed pulmonary veno-occlusive disease (PVOD), which led eventually to the finding of an inborn error of cobalamin metabolism, and he died 7.5 years after his first presentation.[190] The last patient died 12 years after aHUS onset, but not due to the consequences of aHUS.

Discussion

In this study, we found that in 45 children diagnosed with aHUS, genetic disorders in complement-regulating genes encoding *CFH*, *CFI*, *CD46*, *CFB*, *C3*, and *THBD*, as well as the presence of αFH with or without a homozygous deletion in *CFHR1/3*, are linked with clinical presentation, treatment, and outcome. In almost 50% of this pediatric aHUS cohort, a genetic defect in complement-regulating genes was found. This finding is compatible with the previous French pediatric report of Sellier-Leclerc *et al*. and the Italian adult/pediatric registry reported by Noris *et al*.[90, 139] Most mutations (overall 13%) were found in *CFH*, which corresponds to the literature.[71, 72, 90, 139] The number of mutations in *CD46* and *C3* were equal (overall 9%). Fewer mutations (overall 7%) were found in *CFI*, just as seen in the pediatric French cohort. Mutations in *CFB* are relatively rare and according to the literature occur in only 1–2% of patients with aHUS.[29, 72, 90] We identified a genetic aberration in *CFB* in 4.4% of our patients, but it must be mentioned that these two patients were related. Delvaeye *et al*. report that about 5% of patients with aHUS have mutations that impair thrombomodulin function.[73, 90] Remarkably, in our cohort, no aHUS-associated thrombomodulin mutations were found.

Dragon-Durey *et al*. were the first to describe the association between αFH and aHUS, and more recently an association between the presence of αFH and a homozygous polymorphic deletion in Δ*CFHR1/3* was described.[81, 137] In our population, 13% of all patients had αFH, and 83% of these patients (overall 11%) had an additional Δ*CFHR1/3*, which corresponds well to previous published data.[81, 137, 141, 191]

One of the most important observations in this cohort is that 74% of aHUS patients showed gastrointestinal symptoms, of which 25% presented with diarrhea. This clearly shows that

postdiarrheal onset does not exclude the possibility of aHUS. This makes differentiating between typical HUS and aHUS for the general pediatrician sometimes even more difficult and may even result in a delay of adequate treatment in these patients. Also, relapses seem to occur most often after a triggering event, usually an upper airway infection or gastroenteritis. Remarkably, in one patient, aHUS relapsed twice after a cytomegalovirus infection for which daily PE and ganciclovir were initiated.[192] In one other patient, aHUS onset followed a hepatitis B vaccination a few days earlier. The triggering role of vaccination in this patient was confirmed by a relapse shortly after combined anti-diphtheria-pertussis-tetanus-polio vaccination.

In only 18 of the 37 tested patients (49%) were C3 plasma levels reduced, which indicates, as shown by others, that normal C3 levels in aHUS patients do not reflect complement activation on the endothelial cell surfaces. Normal C3 levels also do not rule out the existence of mutations in genes encoding complement-regulating proteins. Breakdown products of C3 activation, such as C3d, as well as soluble complement complex C5b-9 (SC5b-9), might be a better serum marker for complement activation in aHUS patients.

Although at this time results of the use of eculizumab in patients with aHUS look very promising and might replace plasma therapy in the near future, at present, plasma therapy is considered the gold standard treatment in patients with aHUS and should be started within 24 h of diagnosis.[96, 193, 194] However, only 60% of our population received plasma therapy (with or without dialysis). This is due to the fact that in our study, patients were included at the time their DNA was sent to the laboratory between 2001 and 2010, a time when knowledge about aHUS treatment was growing. However, those patients' first or even second HUS episode was sometimes long before that period, when no clear therapy schedules were available. Of the patients treated without plasma therapy, 44% were diagnosed and already treated before 2000. Only in recent years have new treatment guidelines for aHUS been published.[96] Since the latest guideline in 2008, every patient in this study was treated with plasma therapy.

In our cohort, seven patients received a total number of 13 kidney transplants. In five of these seven patients, transplantation was performed before the existence of genetic complement mutations was known and before plasmapheresis as treatment of aHUS was well accepted. Four of these patients had a genetic defect (1x *CD46*, 2x *CFH*, and 1x *CFI/C3*). All except one (*CD46*) graft failed (93%), again demonstrating that kidney transplantation is compromised by the risk of recurrence.[29, 71, 90, 138, 139, 195] Renal transplantation in patients with aHUS should not be performed without DNA analysis for all involved complement genes due to the fact that this leads to a better defined risk profile for relapses.

Currently, aHUS patients having a *CFH* or *CFI* mutation who will have a single kidney transplantation are advised to combine this with prophylactic plasmapheresis and life-long PE, or else these patients are advised to have a combined liver–kidney transplantation with preoperative PE, intraoperative PI, and posttransplant anticoagulation.[195, 196] In the near future, one might expect that lifelong prophylactic treatment with complement inhibitors will replace the use of plasma and /or the combination of plasma with liver and/or renal transplantation in aHUS patients with *CFH* or *CFI* mutations.[193, 194]

In conclusion, in almost 50% of pediatric aHUS patients, a genetic abnormality was found. In 87% of patients, aHUS onset followed a triggering event; 25% of cases presented with diarrhea, which makes differentiating between typical and aHUS difficult. Half of the patients (21/43) had a relapse (67% of them with a genetic anomaly), and all except one renal graft failed, so renal transplantation should not be performed before adequate DNA analysis of the involved complement genes. In the near future, next-generation sequencing methods, such as whole exome or genome sequencing, will more easily facilitate DNA mutation analysis, and new genes will probably be identified that might be associated with aHUS as well. With the use of complement inhibitors in the ongoing trials in aHUS patients, a new era in the treatment of patients with aHUS has begun. It has yet to be demonstrated whether this treatment will improve the overall results in aHUS patients and hopefully reduce the requirement for dialysis and renal transplantations.

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Chapter 7

The challenge of managing hemophilia A and STEC-induced hemolytic uremic syndrome

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Abstract

The hemolytic uremic syndrome (HUS) is a thrombotic microangiopathy leading to acute renal failure in children. In most cases it is triggered by a shiga-like toxin-producing *Escherichia coli* (STEC) infection. Endothelial damage plays a central role in the pathogenesis of disease. Hemophilia A is a genetic disorder leading to factor VIII (FVIII) deficiency, an important factor in the coagulation system. Here we describe a hemophilia A patient that developed HUS due to a STEC O26 infection. Besides acute renal failure, severe gastro-intestinal and neurological complications occurred. Increased amounts of recombinant FVIII (rFVIII) had to be administered during the acute phase of the disease to reach acceptable blood levels of FVIII, to control the hemorrhagic colitis, and to prevent severe neurological complications. The patient's treatment schedule of rFVIII during the HUS period was a big challenge and we cannot exclude that it contributed to the severity of the HUS by enhancing the thrombotic microangiopathic process. The role of factor VIII administration in the severe outcome of disease is discussed.

Introduction

The hemolytic uremic syndrome (HUS), a thrombotic microangiopathy (TMA), is one of the most common causes of acute renal failure in children between two and five years of age in Western Europe.[37] In more than 90%, the disease is triggered by an infection with shiga-like toxin-producing *Escherichia coli* (STEC).[133] Most cases are associated with STEC serotype O157:H7, but other serotypes have been mentioned.[25] Patients have abdominal pains with watery diarrhea, frequently followed by bloody diarrhea, three to eight days after contamination. These symptoms are followed by hemolytic anemia, thrombocytopenia, and acute renal failure, sometimes already within 24 hours.

Here, we report severe renal, gastro-intestinal, and neurological complications due to a STEC O26 infection in a 2-year old hemophilia A patient. Increased amounts of recombinant factor VIII (rFVIII) had to be administered during the acute phase of the disease. To our knowledge, this is the first report of a hemophilia patient that developed STEC-HUS. The treatment of hemophilia in the acute phase cannot be excluded to have contributed to the difficult management of the disease and to the development of severe complications.

Case report

The patient is the first child in a non-consanguineous family. As his mother is a carrier of hemophilia A, FVIII activity was determined in the neonatal period and severe hemophilia A was diagnosed. A spinal-epidural bleeding from C1 till sacral level occurred at the age of one year, after which prophylactic rFVIII therapy of 500 IU three times a week was started.

At the age of two years and 9 months, he developed abdominal pain, vomiting, and bloody diarrhea. Intravenous rehydration was started and due to severe gastro-intestinal blood loss, treatment with rFVIII was increased to twice a day 500 IU (35 IU/kg). An abdominal x-ray demonstrated a dilated stomach, but no signs of ileus, abdominal or retroperitoneal bleeding. After two days he developed oliguria and a blood sample showed all signs of hemolytic uremic syndrome. Fecal samples for STEC detection were taken and the patient was transferred to the university hospital.

On admission, a pale, uncomfortable, somnolent boy with slight peri-orbital and pretibial edema was seen. He was slightly dehydrated, had normal blood pressure (91/59 mmHg), a heart rate of

100-120 beats/min, and a body temperature of 39°C. No respiratory and cardiac abnormalities were seen. Results of laboratory evaluation at admission, including hemoglobin levels, hematocrit levels, thrombocyt count, and white blood cell count are shown in Table 7.1. Abdominal examination showed tenderness by palpation. Conservative treatment for renal failure was started. Serologically FVIII measured on admission was 15% (10 hours after last rFVIII administration), and due to ongoing rectal blood loss, rFVIII dosage was increased to 1000 IU (70 IU/kg) five times a day in the first two days, leading to a FVIII activity of 40 - 102% (Figure 7.1A).

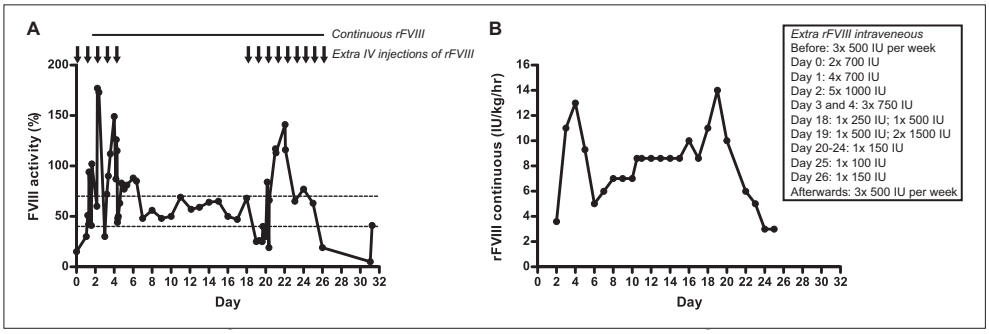

Figure 7.1. Factor VIII activities (A) and doses used in continuous infusion of rFVIII (B) in our patient. Target levels for FVIII activity were 40% to 70%, indicated with the dashed lines. Continuous rFVIII (IU/kg/hr) was administered from day 2 until day 26. To maintain FVIII levels at acceptable levels, in the first days and during the bleeding at the site of the jugular catheter, extra intravenous rFVIII was given (shown with arrows **(A)** and in box **(B)**).

On the second evening he developed a tonic-clonic epileptic insult. Laboratory evaluation showed slightly decreased serum sodium with normal other electrolytes, decreased serum albumin, and elevated urea, creatinine, and glucose (Table 7.1). Blood pressure at that moment was normal (115/71, 85/57, and 106/74 mmHg). A CT-scan cerebrum showed symmetrical ventricles, normal aspects of cerebellum and brain stem, and no signs of hemorrhagic bleeding. Electric encephalogram and cephalorraquid liquid examination were normal. Neurological examination the day after the insult demonstrated a lower level of consciousness, discomfort, hypertonia, abnormal posturing of the head to the right, aphasia, and increased salivation with discomfort. At this time, bilateral symmetric signal intensity changes in the basal ganglia were seen on MRI: a typical finding in HUS patients with neurological complications.[197]

Because of anuria, symptoms of ileus, and severity of neurological condition, continuous veno-venous hemofiltration (CVVH) was initiated, as well as plasmapheresis. No antibiotics were prescribed. Plasmapheresis was stopped after confirmation of a STEC O26 infection. Because of

hemorrhagic colitis, severe neurological condition, hypertension, and bleeding at the exit site of the jugular catheter, all in combination with thrombocytopenia and persistently low FVIII levels in plasma, FVIII treatment was switched to continuous infusion, with a target level of 40-70%. The dosage of continuous rFVIII needed to reach these levels varied between 5 and 14 IU/kg/hr (Figure 7.1B). Erythrocyte transfusions were given eight times. CVVH, combined with antihypertensive treatment, was continued for 18 days, in which renal function and hypertension improved.

Three months after discharge, the boy was diagnosed with sigmoid stenosis, necessitating a sigmoid resection and end-to-end anastomosis. Currently, one year after the acute phase of disease, his creatinine clearance is normal (95 ml/min/1.73m^2), but he still has proteinuria and hypertension treated with an ACE inhibitor. The prophylactic rFVIII therapy of 500 IU three times a week was resumed after discharge. Until now, no FVIII inhibitor had been detected. An intensive rehabilitation program was started and led to a clear improvement of the neurological involvement in our patient. There is still a slight lateralization to the right side of the body with hypertonia and hyperreflexia on the left side.

Table 7.1. Laboratory evaluation at different time points. Day 0 is day of admission to the university pediatric nephrology ward. Normal values for children are shown within brackets.

	Admission	Day 0	Day 1	Day 2	At discharge
serum creatinine (0.17 – 0.51 mg/dL)	2.25	2.71	4.07	4.05	0.66
serum urea (7.0 – 19.6 mg/dL)	32.5	43.7	70.9	75.6	30.2
hemoglobin (9.7 – 14.5 g/dL)	10.6	10.1	9.3	8.4	8.4
hematocrit (0.30 – 0.45 L/L)	0.32	0.30	0.27	0.24	0.25
thrombocytes (120 – 350 x10^3/μL)	65	68	72	82	409
white blood cell count (6.0 – 18.0 10^3/μL)	22.2	25.4	30.2	23.8	8.1
serum sodium (135 – 145 mEq/L)	131	127	128	126	138
serum potassium (3.5 – 4.7 mEq/L)	N/A	4.0	4.2	3.6	4.7
magnesium (1.40 – 2.20 mEq/L)	N/A	1.56	1.84	1.52	2.14
calcium (8.82 – 10.62 mg/dL)	N/A	7.90	7.56	6.53	10.4
serum albumin (3.5 – 5.0 g/dL)	N/A	2.3	1.8	1.9	N/A
glucose (72.1 – 100.9 mg/dL)	N/A	109.9	99.1	151.3	N/A

N/A indicates not available. Conversion factors for units: serum creatinine in mg/dL to μmol/L, ×88.4; serum urea in mg/dL to mmol/L, ×0.357; hemoglobin in g/dL to mmol/L, ×0.6206; hemoglobin in g/dL to g/L, ×10; magnesium in mEq/L to mmol/l, ×0.5; calcium in mg/dL to mmol/L, ×0.2495; serum albumin in g/dL to g/L, ×10; glucose in mg/dL to mmol/L, x0.05551. No conversion necessary for serum sodium in mEq/L and mmol/L, for serum potassion in mEq/L and mmol/L, for thrombocytes in 10^3/μL and 10^9/L, and for white blood cell count in 10^3/μL and 10^9/L.

Discussion

We describe a 2-year-old boy, already known with severe hemophilia A and treated with prophylactic rFVIII, who developed a severe form of HUS due to a STEC O26 infection. The dosage of rFVIII had to be increased enormously to reach acceptable blood levels of FVIII to control the hemorrhagic colitis and a bleeding at the site of the jugular catheter, and to prevent severe neurological complications like intracerebral bleedings. Although his renal function recovered after 18 days of CVVH, proteinuria and hypertension still persist. An intensive rehabilitation program made clear neurological progress, but the boy is still not fully rehabilitated. The question rose if the intensified treatment with rFVIII could have negatively influenced the outcome of this STEC-HUS patient.

Infusion of FVIII results in complex formation with von Willebrand Factor (vWF), the carrier protein of FVIII in plasma. FVIII is released from the complex after activation by thrombin and subsequently participates in the propagation of the coagulation cascade by acting as a cofactor in the conversion of FX to FXa. Factor Xa will activate prothrombin to thrombin, eventually resulting in the formation of a stable fibrin clot. It was demonstrated that the thrombi in the glomeruli of typical HUS patients consist mostly of fibrin.[198] Furthermore, *in vitro* experiments have shown that shiga toxin induces the secretion of vWF from microvascular endothelial cells.[199] Next to its FVIII carrier function, vWF plays an important role in platelet adhesion after vessel wall injury. In this way, the adhesion of platelets will probably lead to increased thrombus formation in the microvasculature of the kidney of HUS patients.

The supraphysiological doses of rFVIII needed in the first days of disease to treat the underlying pathology of hemophilia A together with the release of increased secretion of vWF by the HUS pathology may have contributed further to the TMA. It is known that increased levels of FVIII in children can lead to acquired thrombophilia, a state of hypercoagulability.[200] It therefore may be very plausible that increased amounts of rFVIII contributed to increased thrombosis after shiga toxin-induced endothelial damage in the affected organs (especially intestines and brain). It is also known that hypertension can be responsible for cerebral involvement in HUS patients.[201] Increased FVIII levels are significantly associated with intracranial hypertension[202], and rFVIII administration could have contributed to the encephalopathy by this route as well. Bolus injections together with continuous infusion of rFVIII resulted in diverging FVIII levels (Figure 7.1A), which may have

compromised the disease. Therefore, only continuous infusion might be recommended to avoid a transient overdose of FVIII.

The STEC serotype O26 found in this patient is one of the most common non-O157 STECs causing diarrhea and HUS.[203] Neurological complications are seen often in O26-HUS, although case series disagree on their statistical significance. A study in Scotland showed a significant difference to the prejudice of O26 infection in disease severity and outcome[204], while O26 patients in Austria and Germany did not have more neurological complications [203]. Involvement of the basal ganglia is seen in the majority of the HUS patients with neurological complications and is frequently associated with good clinical outcome[205], as is shown partly in our patient. Genetic aberrations, associated with the atypical form of HUS, were not identified in this patient.

It is known that HUS patients with a combination on admission of dehydration, a longer period of oligoanuria, a WBC count of more than 20 $x10^3/\mu L$, and a hematocrit of more than 0.23 L/L, are at risk for a more severe disease course and even death.[206] Furthermore, in HUS patients with hemorrhagic colitis, renal and neurological complications were more severe than in patients without HC.[207] All above mentioned criteria were present in our patient (Table 7.1), which indicates that his severe condition could be due to all this as well.

Here we have reported a hemophilia patient whom developed HUS after a STEC O26 infection, complicated by severe neurological and gastro-intestinal complications. His treatment schedule of rFVIII during the HUS period was a big challenge. Although the severe complications seen could be due to the specific STEC serotype, hemorrhagic colitis, or the combination of dehydration, oligoanuria, high WBC numbers, and high hematocrit, we cannot exclude that the administration of rFVIII also contributed to the severity of the HUS by enhancing the thrombotic microangiopathic process.

Acknowledgements

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Section II: Complement dysregulation in HUS

Chapter 8

Complement activation patterns in atypical hemolytic uremic syndrome during acute phase and in remission

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Abstract

Atypical hemolytic uremic syndrome (aHUS) is associated with (genetic) alterations in the alternative complement pathway. Nevertheless, comprehensive evidence that the complement system in aHUS patients is more prone to activation is still lacking. Therefore, we performed a thorough analysis of complement activation in acute phase and in remission of this disease.

Complement activation patterns of the aHUS patients in acute phase and in remission were compared to those of healthy controls. Background levels of complement activation products C3b/c, C3bBbP and TCC were measured using ELISA in EDTA plasma. *In vitro* triggered complement activation in serum samples was studied using zymosan-coating and pathway-specific assay. Furthermore, efficiencies of the C3b/c, C3bBbP, and TCC generation in fluid phase during spontaneous activation were analyzed.

Patients with acute aHUS showed elevated levels of C3b/c ($P<0.01$), C3bBbP ($P<0.0001$) and TCC ($P<0.0001$) in EDTA plasma, while values of patients in remission were normal, compared to those of healthy controls. Using data from a single aHUS patient with complement factor B mutation, we illustrate normalization of complement activation during aHUS recovery. Serum samples from patients in remission showed normal *in vitro* patterns of complement activation and demonstrated normal kinetics of complement activation in the fluid phase.

Our data indicate that while aHUS patients have clearly activated complement in the acute phase of the disease, this is not the case in remission of aHUS. This knowledge gives important insight into complement regulation in aHUS and may have an impact on monitoring of these patients, particularly when using complement inhibition therapy.

Introduction

Atypical hemolytic uremic syndrome (aHUS) is a severe renal illness with up to 50% of cases progressing to end stage renal disease (ESRD) and up to 25% of lethal outcomes in the acute phase of aHUS.[3] Complement dysregulation, leading to glomerular endothelial cell damage is considered to be a central element in aHUS pathogenesis and complement inhibition therapy, reported to be successful, has recently been approved for aHUS treatment.[104, 208, 209]

The complement system, a part of the innate immune system, can be activated via three pathways: the classical, the lectin and the alternative. These pathways converge at the cleavage and activation of the central complement component C3. This results in formation of the C3b fragment, which binds zymogen complement factor B (FB) and, in association with properdin, forms the alternative pathway C3 convertase (C3bBbP). C3bBbP cleaves and activates more C3 molecules, leading to formation of the terminal C5b-9 complement complex (TCC) and release of the potent anaphylatoxins C3a and C5a. To protect healthy host tissue, the C3 convertase activity is controlled by complement inhibitors, such as complement factor I (FI), complement factor H (FH) and membrane cofactor protein (CD46/MCP). As a result of C3 convertase inactivation, various C3 degradation products are formed, such as iC3b, C3c and C3dg.[66, 210]

Currently, alternative complement pathway deficiencies are identified in 50-60% of aHUS patients. Genetic variants affecting FH, FI, CD46, C3, and FB, as well as autoantibodies against FH (αFH) are associated with aHUS pathogenesis. Also aberrations affecting genes outside of the complement cascade, encoding thrombomodulin (*THBD*) and diacylglycerol kinase ε (*DGKE*) were reported.[69-71, 73-75, 80, 81, 83, 94, 143, 175, 211, 212] Etiological analysis of patients with aHUS is critically important, especially in renal transplantation, which is frequently required in this patient group. For example, patients that carry mutations in genes encoding FH, FI, or C3 are at higher risk of the disease recurrence in the graft (40-90%), whereas such probability is lower for the aHUS patients carrying CD46 mutations and αFH autoantibodies.[29, 78, 95, 213] Functional analysis of complement activation in aHUS patients remains very limited. C3 and C4 levels, which are used in clinical practice, often show normal values. Recently, a single study reported that C5a and TCC are higher in aHUS than in thrombotic thrombocytopenic purpura (associated not with complement aberrations, but with ADAMTS13 deficiency).[214] However, it is still not clear whether the complement system is more prone to activation in aHUS patients than in healthy individuals.

In this study, we for the first time performed thorough analysis of complement activation in aHUS patients in the acute phase of the disease and in remission. Monitoring of complement activation

dynamics in EDTA plasma and serum samples may provide a simple and important tool in assessment of complement activation in aHUS patients, particularly when complement inhibition therapy is used.

Materials and methods

Study population

The research population consisted of two groups of aHUS patients in acute phase (n=6) and in remission (n=11) of the disease. The acute phase was defined as the presence of hemolytic anemia, thrombocytopenia and acute renal failure. Patients in remission had their last aHUS episode more than one year ago. In addition, from a single aHUS patient with a p.Lys323Glu mutation in FB, samples were taken during two acute aHUS episodes, in following convalescence periods during plasmapheresis (PF) treatment, and in the remission phase at the time of the study. The patients were referred to the Pediatric Nephrology Department of the Radboud university medical center. The control group consisted of healthy adult volunteers. For the controls the following exclusion criteria were applicable: fever, bacterial/viral infection in previous two weeks, chronic illness, inborn or acquired immune disorders, immunosuppressive medication. The study was approved by the institutional review board and was performed in accordance with the appropriate version of the Declaration of Helsinki. Informed consent of all patients and/or their parents as well as of controls was obtained before analysis.

Genetic analysis

Genomic DNA was isolated from peripheral blood leukocytes as described by Miller *et al*.[140] Coding fragments of *CFH, CFI, MCP, C3*, *CFB*, *THBD*, and *DGKE* genes were amplified from genomic DNA by means of PCR. Primer sequences are available upon request. The obtained PCR products included DNA sequences of the individual exons, flanked by the splice donor site and the splice acceptor site. The amplimers were subjected to double-stranded DNA sequence analysis on an ABI 3130 *xl* GeneticAnalyzer (Applied Biosystems, South San Francisco, CA, USA). Sequence analyses were performed using Sequencher 4.8 software (Gene Codes, Ann Arbor, MI USA). Anti-FH autoantibodies were detected as described before.[72]

Sample collection

EDTA blood and serum samples of patients and controls were collected and processed within one

hour after sampling. Samples were aliquoted and stored at -80°C. To avoid *in vitro* complement activation after sampling, EDTA plasma samples were used to compare complement activation in acute phase and in remission of aHUS. Serum samples were used in the *in vitro* complement activation experiments.

Complement activation analyses

The levels of complement activation products C3b and its degraded form C3c (C3b/c), the alternative complement pathway convertase C3bBbP, and the fluid phase terminal sC5b-9 complement complex (TCC) in EDTA plasma were quantified using enzyme-linked immunosorbent assay (ELISA) as described in detail previously by Bergseth *et al*.[215] The international complement standard#2 (ICS#2) was used for quantification of activation products in complement activation units per ml (CAU/ml).[215]

Zymosan-induced activation of serum was performed in 96-well plates coated using 2 µg/well of zymosan A from *Saccharomyces cerevisiae* (Sigma-Aldrich, St. Louis, MO, USA). Complement activation and detection of C3b and TCC deposition on the solid phase was performed as described before.[216]

To analyze the classical, lectin and alternative pathway activation separately, a Wieslab® Complement system Screenkit (Euro Diagnostica, Malmö, Sweden) was used according to the manufacturer's protocol.

In a kinetic activation analysis, serum samples were diluted 1:2 in Dulbecco's phosphate buffered saline with $MgCl_2$ and $CaCl_2$ (Sigma-Aldrich) and incubated at 37°C with gentle agitation, samples were collected at 0, 10, 20, 30 and 60 minutes of incubation and immediately placed on ice. To stop the complement activation, EDTA (Sigma-Aldrich) was added to a final concentration of 20 mM. Complement activation products were quantified as described above for EDTA plasma samples.

Statistical analysis

Statistical analysis was done using unpaired two-tailed t-test. Statistically significant differences with $P<0.05$ are indicated.

Results

Genetic background of the aHUS patients

For this study, samples from two groups of patients in the acute phase (n=6) and in remission (n=11) were available. The patients in both groups were screened for the presence of pathogenic genetic variations in genes encoding FH, FI, CD46, C3, FB, THBD, and DGKE; furthermore, presence of αFH autoantibodies was analyzed. In the acute phase aHUS group, two of the patients carried heterozygous genetic variants in *C3* or *CD46* and one had αFH autoantibodies, while three patients had aHUS of undefined etiology (Table 8.1). In the aHUS remission group, six patients carried heterozygous changes in *CFH*, *CFI*, *C3* or *THBD*; in five patients no genetic aberrations or αFH autoantibodies were found (Table 8.2). Familial and idiopathic aHUS patients were present in both groups (Table 8.1 and Table 8.2).

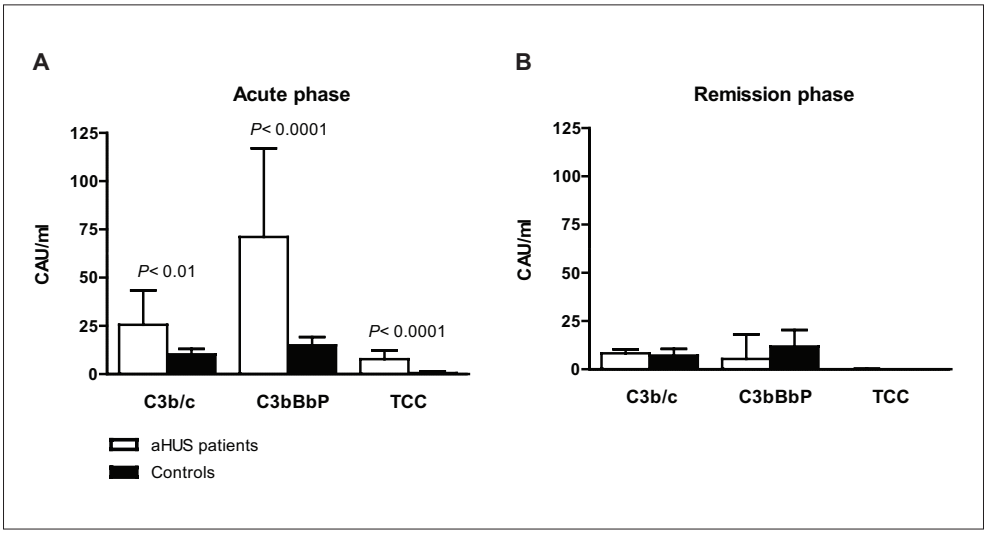

Figure 8.1. Plasma complement activation products in patients with aHUS in acute phase and in remission. EDTA plasma samples of six aHUS patients in acute phase (**A**) and of 11 patients in remission phase (**B**) were analyzed for the levels of C3b/c, C3bBbP and TCC. The values were compared to those of 19 healthy controls. Data were quantified using the international complement standard#2 (ICS#2) in complement activation units per ml (CAU/ml) and are presented as mean+standard deviation. Values that were statistically different from those of healthy controls (P<0.05) are indicated.

Complement activation in acute phase and in remission of aHUS

First, we analyzed complement activation in the acute phase and in remission of aHUS. To this end, we measured concentrations of complement activation products C3b/c, C3bBbP, and TCC in EDTA plasma of aHUS patients and compared the values to those of healthy controls (n=19). These assays

reflect activation of the alternative complement pathway, which is implicated in aHUS pathogenesis. In the acute phase of the disease, we observed significantly elevated levels of C3b/c, C3bBbP, and TCC (Figure 8.1A). In four of the six patients in the acute phase, all three activation products were elevated; in the other two patients, two of the three activation product were elevated, indicating ongoing complement activation in all of these patients (Table 8.3). The values of C3bBbP and TCC were increased in all six patients, including the three patients with normal serum C3 levels in the acute phase. On the other hand, patients in remission showed C3b/c, C3bBbP, and TCC values that were completely comparable to those of healthy controls (Figure 8.1B).

From a single female patient, carrying the well-characterized p.Lys323Glu mutation in FB[74], samples taken during two acute aHUS episodes, following PF treatments, and in remission were available at the time of the study (Table 8.4). Increased concentrations of all three complement activation markers (first episode) and C3b/c and C3bBbP (second episode) were detected in the acute phase. Complement activation levels considerably decreased during PF treatments and were completely normalized in remission.

Together, our data indicate that in acute aHUS episodes complement activation is taking place, but returns to the level of healthy controls during remission.

Induced complement activation in serum samples of aHUS patients in acute phase

Ongoing complement activation in the acute phase of aHUS may lead to complement consumption as partially seen from the C3 serum levels (Table 8.3). This may cause diminished capacity for complement activation. Next, we tested this hypothesis using a pathway-specific activation assay, widely used in clinical laboratories (Figure 8.2). Our results indicate that the classical and lectin pathway activation profiles are not different between the acute phase patients and healthy individuals. In the alternative pathway experiment, only one of the acute phase patients (P6a) demonstrated activation outside of the normal range.

Induced complement activation in serum samples of aHUS patients in remission

Next, we analyzed whether, when triggered, serum samples of aHUS patients in remission are able to reach higher complement activation levels than the samples of healthy controls. To this end, we performed complement activation on the zymosan-coated surface. Zymosan is able to directly activate the lectin and the alternative complement pathway and also activates the classical pathway when anti-zymosan antibodies are present in serum.[217] We quantified C3b and TCC deposition on zymosan-coated surfaces in response to the activation of serum samples. No significant differences

Table 8.1. Clinical and genetic data available for patients in the acute phase of aHUS.

Patient	aHUS pathogenic change	Gender	Age at onset/ time of study [a]	Treatment	Outcome
P1a [b]	C3: p.Arg161Trp[94, 175]	Male	1st episode: 6 years; 2nd episode: 12 years	Plasma therapy	Remission
P2a	None	Female	7 years	Plasma therapy	Remission
P3a	Anti-FH autoantibodies[81]	Female	11 years	Plasma therapy, hemodialysis	Remission
P4a	MCP: p.Asp271_Ser272del[143]	Male	1st episode: 4 years; 2nd episode: 8 years	Plasma therapy	Remission
P5a [b]	None	Male	1st episode: 7 months; 2nd episode: 2 years	Plasma therapy, eculizumab	Remission under eculizumab
P6a	None	Male	5 years	Plasma therapy, CVVHDF [c]	Remission

[a] Age at onset of the latest episode for these patients is equal to the age at the time of the study. First episodes of patients P1a, P4a and P5a resolved in (nearly) complete remission without need of a renal transplantation; [b] Familial aHUS; [c] CVVHDF stands for continuous veno-venous hemodiafiltration.

Table 8.2. Clinical and genetic data available for patients in the remission phase of aHUS.

Patient	aHUS pathogenic change	Gender	Age at first onset/ time of study	Treatment	Outcome	Transplantation history
P1r [a]	FH: p.Arg1206Cys[212]	Male	22 years / 27 years	Plasma therapy, hemodialysis	remission	None
P2r [a, b]	FH: p.Arg1206Cys[212]	Female	21 years / 48 years	Plasma therapy, hemodialysis	ESRD	aHUS in graft
P3r	FI: p.Arg474Stop[70]	Female	49 years / 57 years	Plasma therapy, hemodialysis	ESRD	No recurrence
P4r [a]	C3: p.Arg161Trp[94, 175]	Male	52 years / 59 years	Plasma therapy, hemodialysis	ESRD	No recurrence
P5r [a]	C3: p.Arg161Trp[94, 175]	Female	23 years / 35 years	Plasma therapy	ESRD	No recurrence
P6r	THBD: p.Ala43Thr[73]	Female	2 months / 5 years	Antibiotics, CVVH [c]	remission	None
P7r	None	Male	24 years / 29 years	Hemodialysis	ESRD	None, on the waiting list for transplantation
P8r	None	Female	3 years / 23 years	Hemodialysis	ESRD	aHUS in graft
P9r	None	Male	1 years / 13 years	Peritoneal dialysis	ESRD	aHUS in graft
P10r	None	Female	15 years / 24 years	Hemodialysis	ESRD	No recurrence
P11r [d]	None	Male	8 years / 17 years	Blood transfusions	ESRD	No recurrence

[a] Familial aHUS.; [b] Mother of P1r; [c] CVVH stands for continuous veno-venous hemofiltration; [d] Full spectrum aHUS developed during cyclosporine treatment for familial steroid resistant nephrotic syndrome.

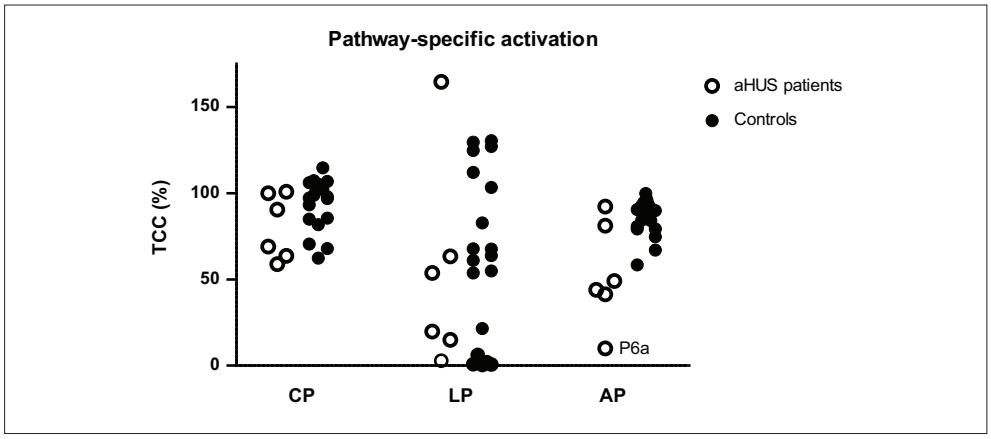

Figure 8.2. Pathway-specific activation of serum samples from acute aHUS patients. Specific activation of the classical (CP), the lectin (LP) and the alternative (AP) pathway of complement was measured with TCC deposition as readout. Data are presented as percentage of activation of the positive control provided with the assay. Low alternative pathway activation value in sample of patient P6a is indicated.

between remission phase aHUS samples and control samples were observed by using zymosan-induced complement activation (Figure 8.3A).

Furthermore, we performed pathway-specific analysis to quantify activation of the classical, lectin, and alternative pathway separately (Figure 8.3B). This analysis also did not reveal significant differences between complement activation in aHUS patients in remission and in healthy controls. These data indicate that complement activation in serum samples of aHUS patients in remission is not triggered to a higher level than in samples of healthy controls.

Kinetics of complement activation in serum samples of aHUS patients in remission

So far, no differences in complement activation between patients in remission and controls were observed. Finally, we analyzed whether serum samples of aHUS patients in remission have faster activation kinetics compared to samples of healthy controls. Thus, we studied the kinetics of spontaneous complement activation in fluid phase. Serum samples were incubated at 37°C to induce spontaneous alternative pathway complement activation, after which concentrations of C3b/c, C3bBbP and TCC were analyzed at various time points (Figure 8.4). No statistically significant differences between patients and controls were observed.

These results indicate that there is no difference in complement activation kinetics between aHUS patients in remission and healthy controls.

Discussion

Complement dysregulation is an established hallmark of aHUS. In this study we, to our best knowledge, for the first time present a comprehensive analysis of complement activation in blood of aHUS patients in the acute phase and in remission.

We observed a clear activation of the alternative complement pathway in the acute phase group, as revealed by significant increases in C3b/c, C3bBbP, and TCC in EDTA plasma. The levels of these complement activation markers in aHUS patients in remission, however, were normal. Moreover, when analyzed in a single aHUS patient with a FB mutation, complement activation was clearly detected in both aHUS episodes, but not in remission. Therefore, we suggest that in remission, complement activation in blood of aHUS patients normalizes to the levels of healthy individuals. As our experiments included a limited number of patients, studies in larger cohorts should be performed in the future.

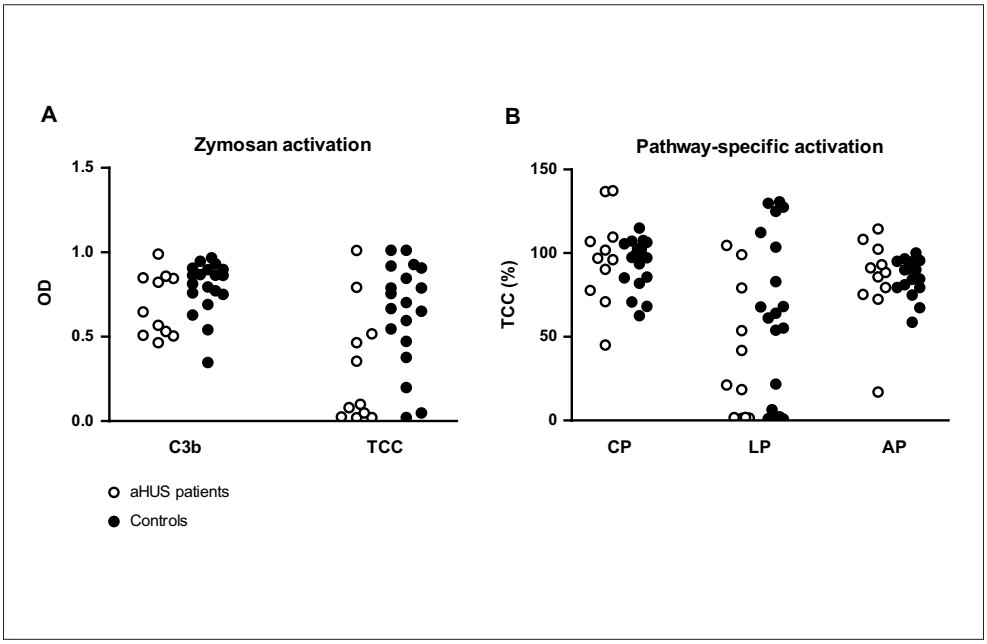

Figure 8.3. Induced complement activation of serum samples from aHUS patients in remission. (**A**) Serum samples of 11 patients in remission phase of aHUS and of 19 healthy controls were analyzed for complement activation in zymosan-coated wells, using C3b and TCC deposition as readout. Results are given as absorbance units of optical density (OD). (**B**) Specific activation of the classical (CP), the lectin (LP) and the alternative (AP) pathway of complement was measured with TCC deposition as readout. Data are presented as percentage of activation of the positive control provided with the assay.

Table 8.3. Complement assessment of patients in the acute phase of aHUS. Values outside of the normal range are indicated in bold.

Patient number	C3 (700-1500) [a]	C4 (100-400) [a]	C3b/c (<15.8) [b]	C3bBbP (<23.5) [b]	TCC (<2.2) [b]
P1a	1110	323	**57.82 (↑)**	**148.7 (↑)**	**7.85 (↑)**
P2a	1130	300	**18.15 (↑)**	**29.3 (↑)**	**15.16 (↑)**
P3a	**639 (↓)**	292	12.25	**46.59 (↑)**	**5.39 (↑)**
P4a	**657 (↓)**	164	**34.25 (↑)**	**67.48 (↑)**	**2.56 (↑)**
P5a	927	205	11.28	**34.74 (↑)**	**4.84 (↑)**
P6a	**620 (↓)**	250	**19.63 (↑)**	**99.95 (↑)**	**10.94 (↑)**

[a] Normal serum concentration range (mg/l) defined by the standard clinical protocol; [b] Normal concentration range (CAU/ml) defined by this study as mean+ 2 standard deviations of control values (n=19).

Table 8.4. Complement assessment of a single female patient during two acute aHUS episodes. Samples were collected in convalescent periods after five (first episode) and 10 (second episode) plasmapheresis (PF) sessions and in remission at the time of the study. The patient recovered without need of a renal transplant and carries the pathogenic p.Lys323Glu mutation in FB[74]. Values outside of the normal range are indicated in bold.

Disease phase	Age (years)	C3b/c (<15.8) [a]	C3bBbP (<23.5) [a]	TCC (<2.2) [a]
First acute episode	2.5	**155.17 (↑)**	**61.58 (↑)**	**2.88 (↑)**
After 5 PF sessions	2.5	**21.57 (↑)**	19.87	1.3
Second acute episode	3.5	**116.16 (↑)**	**115.49 (↑)**	0.74
After 10 PF sessions	3.5	**22.82 (↑)**	10.82	0.67
Remission phase	21	10.33	6.00	0.46

[a] Normal concentration range (CAU/ml) defined by this study as mean+ 2 standard deviations of control values (n=19).

activation might take place at the surface, but not close enough for the TCC to be inserted and thereby generating a fluid phase complex, as typically seen by artificial surfaces such as hemodialysis membrane.[218, 219]

Our data do not exclude the possibility of ongoing complement activation on the cell surface in remission, that could be experimentally detected using sheep erythrocytes or endothelial cells and that was outside of scope of this study.

We found that in a single aHUS patient with a FB mutation, complement activation was clearly detected in both aHUS episodes, but not in remission. Previously, complement activation in a single aHUS patient with the FH mutation p.Ser1191Leu was monitored before and after infusion of fresh frozen plasma.[220] Only limited effect of plasma infusion on complement activation was observed using a hemolytic alternative pathway assay and no difference was observed in a pathway-specific screen. The assays used in this previous study reflect a resultant of several factors, including the amount of native components present, which not necessarily reflect the degree of ongoing activation. This is reflected by the activation product assays we have used. Consistently with these

previous data, all but one patient in our acute phase group, demonstrated normal activation in pathway-specific assay, compared to a control group (Figure 8.2).

Further, we analyzed whether complement activation in serum samples of remission patients can be triggered more efficiently than in samples of healthy controls. Our data indicate that both zymosan-induced activation and pathway-specific activation of aHUS serum samples give results similar to those of healthy controls. Also results of kinetic analysis in the fluid phase appeared to be comparable in aHUS remission patients and in controls.

These findings are indeed intriguing, as six out of 11 patients carry aHUS-predisposing genetic aberrations, which are expected to cause more efficient complement activation. Patient P1r and patient P2r carry the p.Arg1206Cys substitution in the *CFH* gene. Previously, the p.Arg1206Ala variant was characterized in functional studies using recombinant FH19-20 fragment.[212] In this work, impaired binding of a FH19-20 fragment carrying p.Arg1206Ala to C3b/d and mouse glomerular endothelium was shown. We expect p.Arg1206Cys to have a similar effect on protein function.

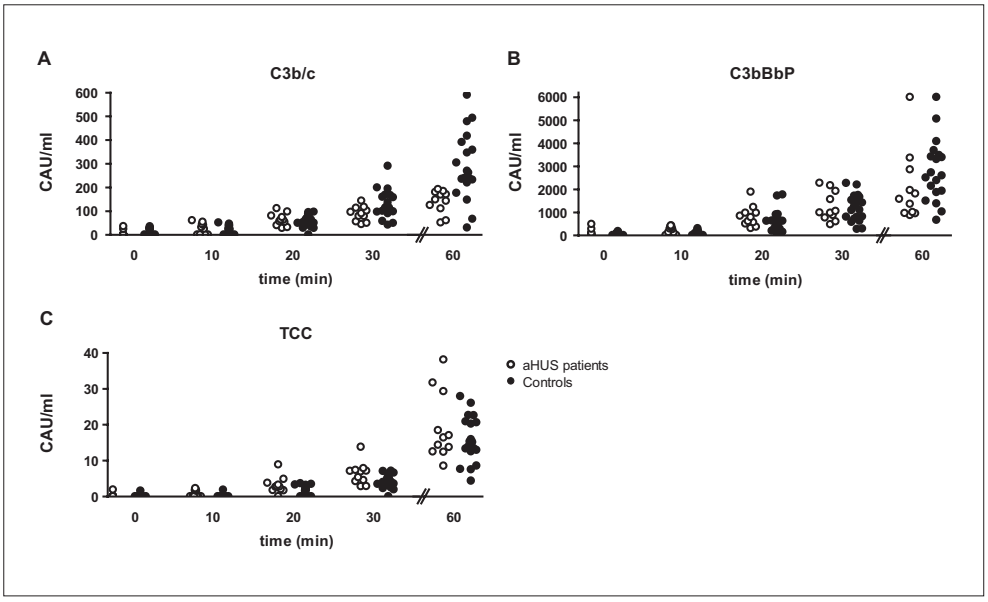

Figure 8.4. *In vitro* kinetics of spontaneous complement activation in the serum samples of aHUS patients in remission. Samples of 11 aHUS patients in remission and of 19 healthy controls were incubated at 37°C with gentle agitation. Samples were collected at 0, 10, 20, 30 and 60 minutes of incubation and levels of C3b/c (**A**), C3bBbP (**B**) and TCC (**C**) were analyzed. Data were quantified using international complement standard#2 (ICS#2) and are presented as complement activation units per ml (CAU/ml).

Patients P4r and P5r carry the C3 variant p.Arg161Trp that was previously reported by us and others and has negative impact on FH and CD46 binding and increases the affinity to FB.[94, 175] Furthermore, thrombomodulin, a component of the coagulation system, was previously reported to regulate inactivation of C3b *in vitro*. The p.Ala43Thr change (P6r) was shown to negatively affect this function.[73] Particularly interesting is the FI mutation in patient P3r, which leads to a stop codon in the serine protease domain that is important for C3b and C4b inactivation.

Absence of a phenotype in remission phase patients might be in line with relatively mild nature of often heterozygous aHUS changes. Penetrance (the chance to acquire aHUS) is estimated to be approximately 60% for aHUS variants. It is suggested that next to a genetic variant or αFH autoantibodies, presence of aHUS-predisposing single nucleotide polymorphisms and external stimuli, such as infections, surgeries or use of medication, is needed for development of aHUS.[221]

In this study, low serum C3 levels were found only in three acute phase patients, indicating complement activation. Moreover, only one patient showed aberrant alternative pathway activation in a pathway-specific activation assay. However, the activation markers, which were elevated in all patients, may provide a more promising tool for the detection of complement activation in aHUS.

The fact that the samples from patients in remission of aHUS display normal complement activation pattern in blood has an important clinical implication. This indicates that in remission phase, aHUS patients are likely to have the same low complement activation levels as that of healthy controls. Therefore, these assays may be used in early detection of relapse of aHUS in the future. Recently, the complement component C5 inhibitor eculizumab (Soliris®) was approved for treatment of aHUS.[104, 208, 209] Patients in the acute phase of aHUS that undergo treatment with complement inhibition therapy can be monitored for the levels of complement activation markers (C3b/c, C3bBbP, and TCC). The value of these markers in patients undergoing eculizumab treatment will be studied in the near future, which may lead to the development of individualized therapy based on the levels of complement activation.

In conclusion, we demonstrated that complement activation can be clearly shown in blood of aHUS patients during acute episodes of the disease and that levels of complement activation markers in the fluid phase completely return to normal during remission. No change in complement activation profile as compared with controls was seen when complement was triggered *in vitro* in the serum

samples of aHUS patients in remission. These assays may be very helpful in the diagnostic setting to monitor complement dysregulation during aHUS attacks, especially when using complement inhibition therapy.

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Chapter 9

Serological and genetic analysis of the complement system in children with hemolytic uremic syndromes

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Submitted.

Abstract

The role of the complement system in the atypical form of HUS (aHUS) has been investigated tremendously in recent years. Genetic and acquired aberrations have been identified in aHUS patients, resulting in a dysregulation of the complement system. It is known that HUS-associated bacteria, like shiga-toxin producing *Escherichia coli* (STEC) and *Streptococcus pneumoniae* (SP), can evade the complement system by binding complement regulators. We hypothesize that a dysregulation of the complement system has an important role in the pathogenesis of STEC-HUS and SP-HUS as well.

Serological and genetic profiles of the alternative complement pathway were prospectively determined in the acute and convalescent phase of disease in 38 HUS patients (27 STEC-HUS patients, 10 aHUS patients, and one SP-HUS patient). To compare the serological profiles to those of age-matched controls, 90 healthy children (aged 5 months – 18 years) were included in the study as well.

In all HUS groups, the alternative pathway was activated in the acute phase of disease, but not in remission. The C3d/C3 ratio displayed the best discrepancy between acute and convalescent phase and between STEC-HUS and aHUS, and might therefore be used as a biomarker in diagnosis and disease monitoring. A genetic complement aberration or the presence of autoantibodies against Factor H was present in 40.5% of the patients, including STEC-HUS and SP-HUS patients. Both patients with and without proven genetic aberrations or autoantibodies had altered serological complement profiles in the acute and/or convalescent phase of disease. The reason for the identified complement activation in infection-induced HUS is not completely clear yet. A prospective study is needed to investigate differences in complement activation in the acute phase of disease and in remission between patients that develop HUS after an infection with STEC or SP and those that do not.

Introduction

The hemolytic uremic syndrome (HUS) is a rare and severe thrombotic microangiopathy (TMA), characterized by the trias hemolytic anemia, thrombocytopenia, and acute renal failure.[1] In most cases, HUS is seen in children between two and five years of age and is often preceded by watery or bloody diarrhoea. It is the most common cause of acute renal failure in infants and children in Western Europe.[37] In more than 90% the disease is triggered by shiga-like toxin-producing *Escherichia coli* (STEC).[133] The outcome of STEC-HUS is relatively good, with ~75% complete recovery and sequelae like proteinuria and hypertension in the remaining. Non-STEC-HUS is seen in 5% to 10% of all pediatric HUS cases, can appear at any age and may be sporadic or familial. These patients have a less favourable prognosis: up to 50% of these so-called atypical cases progress to end-stage renal disease (ESRD) and 25% of the cases may result in death during the acute phase of the disease.[29, 109] A wide variety of triggers for non-STEC-HUS have been identified, including dysregulation of the complement system (aHUS), various non-enteric infections (*Streptococcus pneumonia*), viruses (*HIV*), malignancies, drugs, transplantations, pregnancy, and other underlying medical conditions including systemic diseases.[3, 171, 173]

The human complement system is part of innate immunity and consists of more than 40 plasma and membrane-associated proteins. The most important roles of the complement system are the recognition of pathogens (opsonization), the activation and chemotaxis of leukocytes, and the induction of cell lysis by incorporation of the membrane attack complex.[66] Three activation pathways have been recognized: the classical pathway, the lectin pathway, and the alternative pathway. In the alternative pathway complement component C3 is spontaneously activated at a very low rate to C3a and C3b. Factor B can bind to C3b to form the C3 convertase C3bBb, which is stabilized by properdin and can cleave more C3 molecules. At the surface of normal host cells, complement activation is regulated by the inactivation of C3b and/or dissociation of C3 convertase by regulators in the fluid phase (complement factor H [FH] and factor I [FI]) or by membrane-bound regulators (membrane cofactor protein/CD46 [CD46] and complement receptor 1).[66]

The dysregulation of the alternative pathway of the complement system plays an important role in the pathogenesis of aHUS. In the past years, DNA mutation analysis of genes encoding complement proteins in patients with aHUS have clearly demonstrated that in 50-60% of the patients mutations are found in FH, FI, CD46, FB, and C3.[70-72, 74, 75, 94, 141] A subgroup of patients with aHUS has been described as having antibodies against FH (αFH) in combination with a polymorphic homozygous

deletion of complement factor H related proteins 1 and 3 (CFHR1/3).[81, 82, 137] Next to the mentioned genes, mutations have been identified in three genes encoding coagulation proteins (*THBD*, *DGKE*, and *PLG*).[73, 83, 85]

Recent *in vitro* studies have shown that the microbial pathogens associated with HUS or their toxins can activate the complement system and bind complement proteins, in particular the most important regulator factor H, thereby providing the organism with a mechanism to protect itself against complement activation.[113, 116, 127] Based on the similarity of the clinical manifestations of complement-mediated and infection-induced HUS and the knowledge that bacteria can use complement regulators to survive in the host, we hypothesized that also in patients with HUS caused by an infection with Shiga-like toxin producing *E. coli* (STEC-HUS) and *Streptococcus pneumonia* (SP-HUS), a dysregulation of the complement system has an important place in the pathogenesis.

Anecdotal reports showed low C3 levels and increased complement activation products in children with STEC-HUS and SP-HUS.[65, 91, 110-112, 222] In 2009, a report was published about elevated plasma levels of complement fragments of the alternative pathway (only FB and TCC were measured) in STEC-HUS patients during the first month after onset of the disease, but a small research population was used (seventeen patients) and only four non-age-matched control patients were included.[111] These results were confirmed in a Swedish cohort of 10 STEC-HUS patients, in which C3a and TCC levels were increased in the acute phase of disease and normalized in remission.[112] The use of the complement inhibitor eculizumab in STEC-HUS, however, is still questionable. Even though individual patients can respond rapidly with efficient recovery, in larger cohorts, no significant differences were seen on mortality or morbidity with the use of eculizumab in STEC-HUS patients.[46, 47, 119, 120]

To answer the question if alterations in the alternative complement pathway are common in patients with all forms of HUS, we prospectively determined genetic and serological complement profiles in serum and plasma of children with STEC-HUS, SP-HUS, or aHUS in the acute and in the convalescent phase of the disease. At the moment, in most studies, control ranges for adults are used to interpret results of serological complement tests in children, which is an important shortcoming in the correct interpretation of the results. There are only anecdotal reports of serum complement levels in healthy infants and children, of which most have been published more than

two decades ago, using different in-house and mostly outdated techniques.[223-228] We therefore screened complement profiles in age-matched healthy Dutch infants and children, which have been collected in a strict protocolled way, to obtain reliable reference intervals for the interpretation of our studies in HUS children.

Materials and methods

Patients and controls

Newly diagnosed children (0-18 years) with STEC-HUS, SP-HUS, or aHUS, referred to the pediatric nephrology ward of all eight academic hospitals in The Netherlands between August 2010 and September 2013, were eligible to enroll in the study. EDTA plasma and/or serum samples were collected before the initiation of therapy (acute phase sample) and 14-28 days later (convalescent phase sample), preferably when no signs of TMA were present anymore.

To obtain a more reliable control population, 90 pediatric individuals of different ages were enrolled in the study to assess the correlation between the complement system and age. Exclusion criteria were: fever (>38.5°C), signs or symptoms of infection (bacterial or viral), chronic illness, immune suppressive medication, acquired or congenital immune deficiencies, younger than two days, intensive ventilation, surgical interventions in last three days.

The study was approved by the Medical Ethical Committees of all academic hospitals in which patients were enrolled. The Medical Ethical Committee of the Radboud university medical centre approved the inclusion of control individuals (METC 2010/062). Informed consent for the sample collection was obtained before enrollment.

Sample collection

For serological complement profiling, EDTA blood samples were placed on ice immediately after collection and were processed within one hour (10 min, 2500g, 4°C); whole blood samples were allowed to coagulate for 45-60 minutes before processing (10 min, 2500g, 4°C). Serum and EDTA plasma samples were stored at -80°C in aliquots.

For DNA analysis, genomic DNA was isolated from peripheral blood leukocytes according to established protocols. From one STEC-HUS patient, no material was available for genomic DNA isolation.

Genetic analysis of genes encoding

Genomic DNA was amplified for *CFH* [National Centre for Biotechnology Information (NCBI) RefSeq NM_000186.3], *CFI* (NM_000204.3), *CD46* (NM_002389.4), *C3* (NM_000064.2), and *CFB* (NM_001710.5) by means of PCR, as described before.[72] Primer data are available upon request. Fragments included DNA sequences of the individual exons and the splice donor and acceptor site. Amplimers were subjected to double-stranded DNA sequence analysis on an ABI 3130 *xl* Genetic Analyzer (Applied Biosystems). Sequence analysis was performed using Sequencher 4.8 software. Nonsynonymous aberrations were checked in literature and for presence in the Exome Variant Server (http://evs.gs.washington.edu/EVS/) and an in-house database, which both contain results of whole exome sequencing of >5000 individuals.

Presence of autoantibodies against factor H and anti-O157 LPS antibodies

Serum samples were tested for the presence of αFH by means of enzyme-linked immunosorbent assay (ELISA), as described previously.[229] A positive control sample was obtained via Dr. Dragon-Durey (Paris, France). ELISA tests were also performed on serum samples for the presence of anti-O157 LPS antibodies, as described previously.[230-232] LPS from *Escherichia coli* O157:H7 for coating was obtained via List Biological Laboratories (Campbell, CA, USA).

Serological complement profiling of the alternative complement pathway

The C3 concentration was determined by nephelometry (BN™ II System, Siemens Healthcare Diagnostics, Erlangen, Germany) using reagents from Beckman Coulter Inc (Brae, CA, USA); levels were standardized against the ERM-DA470k/IFCC serum.[233] Levels of FI and FH were determined by radial immunodiffusion. For FI, a goat antiserum raised against purified human factor I was used. Calibration was based on serial dilutions of a normal human serum and expressed as percentage of the value in this standard human serum (% NHS). For FH, a rabbit antiserum raised against purified human factor H was used. Calibration was based on serial dilutions of a normal human serum with a known concentration of FH (in mg/L). To analyze alternative complement pathway activity, a commercially available ELISA kit for total functional assessment of the alternative pathway (AP) was used according to the manufacturer's protocol (Euro Diagnostica, Malmö, Sweden).

Levels of the C3 degradation products C3d and C3b/c, and the alternative complement pathway convertase C3bBbP were quantified in EDTA plasma by means of ELISA, as previously described in detail.[215, 234] As the initial C3 concentration may influence the C3d level, the C3d/C3 ratio was calculated as an extra marker of alternative complement activation independent of the

concentrations of the individual molecules. For this matter, C3d levels were multiplied by 1000 and divided by C3 levels. The fluid phase terminal complement complex (TCC) was measured using a commercially available ELISA kit (Hycult Biotech, Uden, The Netherlands), according to the manufacturer's protocol.

Statistical analysis

A linear regression analysis was performed for the control group to investigate the possible correlation between age and complement profiles. For each investigation, a D'Agostino-Pearson normality test was executed to assess whether the controls were sampled from a Gaussian distribution. When controls were normally distributed, a one-way ANOVA with a Dunnett post-test was executed to analyze differences between controls and independent patient groups; in case controls did not pass the normality test, a one-way ANOVA with a Dunns' post-test was performed. A Mann-Whitney test was used to investigate the difference in age between STEC-HUS and aHUS patients and to compare between the acute and convalescent phase in these independent patient groups. To compare C3d/C3 ratio's between patient groups in the acute phase of disease, a Pearson's chi-square test was performed. All statistical analyses were performed using GraphPad PRISM software (version 5.03 for Windows, GraphPad Software), except for the Pearson's chi-square test, which was executed in SPSS (version 20, IBM).

As only one SP-HUS patient was included, no statistical analysis could be performed for this patient group.

Results

Clinical characteristics of included patients

Thirty-eight patients from six university medical centers in The Netherlands were enrolled in this study between 2010 and 2013: 26 STEC-HUS patients, 11 aHUS patients, and one SP-HUS patient.

Epidemiological features and genetic results for all patients and clinical characteristics (presenting symptoms, treatment, and outcome) for 35 patients (92.1%) are enlisted in Table 9.1. The clinical course of two patients (P1 and P6) has extensively been described before.[235, 236] The mean age of all patients at presentation was 6.5 years old (6.6 years for STEC-HUS; 6.5 years for aHUS; 0.6 years for SP-HUS). No significant difference was seen in age at presentation between pediatric STEC-HUS and pediatric aHUS patients (P=0.9875; Figure 9.1). Even though in literature it is stated that STEC-HUS is

usually diagnosed in children than between 2 and 5 years of age, half of the STEC-HUS patients (13/26; 50.0%) was older than 5 years (range 0.4-17.8 years).

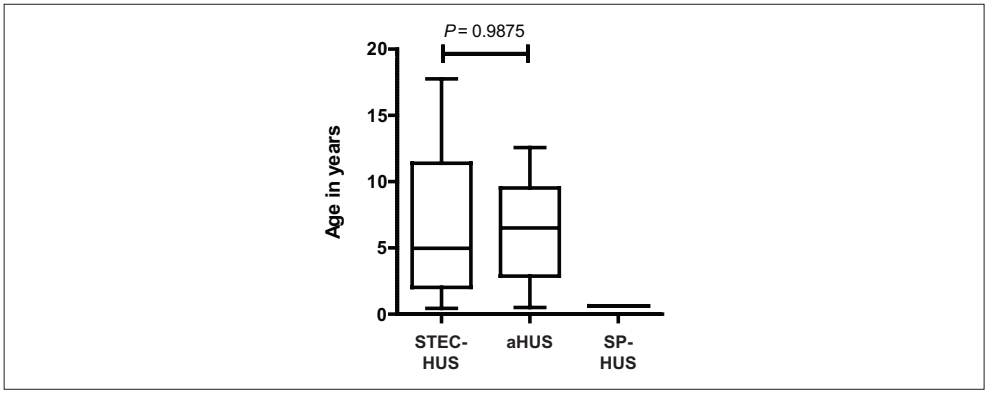

Figure 9.1. Age distribution of STEC-HUS, aHUS, and SP-HUS patients at time of diagnosis. No statistically significant difference is seen between STEC-HUS and aHUS patients. No statistics could be performed for SP-HUS (n=1).

Over 60% of the STEC-HUS cases (16/26) was preceded by an infection with STEC O157; the remaining cases were infected with STEC O26, O5, or O104. In 4 patients (15.4%), culture and/or PCR was positive for STEC-infection, but no serotype could be identified; these patients were all negative for anti-O157 LPS antibodies. All STEC-HUS patients presented with (bloody) diarrhea and/or gastro-intestinal complaints; one patient also had a upper respiratory tract infection. Neurological complications were seen in seven STEC-HUS patients (7/23; 30.4%). Two of these patients had other extra-renal complications as well: one patient suffered from chronic venous insufficiency thrombosis during the HUS episode and the other was diagnosed with sigmoid stenosis due to HUS three months after discharge, for which a sigmoid resection and end-to-end anastomosis was needed.[235] Dialysis was needed in 65.2% (15/23) of the STEC-HUS patients (average duration: 16 days); the other patients recovered without the need for renal replacement therapy. Two STEC-HUS patient were treated with plasmapheresis next to dialysis. In one of them, plasmapheresis was indicated because of anuria, symptoms of ileus, and severity of neurological condition; plasmatherapy was stopped when an STEC O26 infection was confirmed. Two STEC-HUS patients were treated with eculizumab: one STEC O104 patient was treated for eight weeks in the ad hoc off-label trial during the German outbreak, the other had such unusual presentation (12 years old, upper respiratory tract infection, no bloody diarrhea) that aHUS was suspected and one gift of eculizumab was given before anti-O157-LPS antibodies were identified. The majority of the STEC-HUS patients has a good

Table 9.1. Epidemiological and clinical features of included patients.

Feature	All patients	STEC-HUS	aHUS	SP-HUS
Number of patients	38	26 (68.4%)	11 (28.9%)	1 (2.6%)
Gender	20 M : 18 F	13 M : 13 F	7 M : 4 F	1 F
Age (yr) [Mean ± SD (range)]	6.5 ± 4.9 (0.4-17.8)	6.6 ± 5.3 (0.4-17.8)	6.5 ± 3.8 (0.5-12.6)	0.6
Serotype	Not applicable	O157: 16 (61.5%) O26: 4 (15.4%) O5: 1 (3.8%) O104: 1 (3.8%) Not serotyped, but α-O157 negative: 4 (15.4%)	Not applicable	Not performed
Presentation				
Diarrhea	14 (35)	12 (23)	2 (11)	0 (1)
Bloody diarrhea	9 (35)	9 (23)	0 (11)	0 (1)
Gastro-intestinal symptoms without diarrhea	8 (35)	2 (23)	6 (11)	0 (1)
Upper respiratory tract infection	4 (35)	1 (23)	3 (11)	0 (1)
Oligo/anuria	14 (35)	8 (23)	6 (11)	0 (1)
Acute renal insufficiency	1 (35)	0 (23)	1 (11)	0 (1)
Headache	1 (35)	0 (23)	1 (11)	0 (1)
Meningitis	1 (35)	0 (23)	0 (11)	1 (1)
Extra-renal complications				
None	27 (35)	16 (23)	11 (11)	0 (1)
Yes	8 (35)	7 (23)	0 (11)	1 (1)
Neurological	*8 (35)*	*7 (23)*	*0 (11)*	*1 (1)*
Intestinal (sigmoid stenosis, peritonitis)	*2 (35)*	*1 (23)*	*0 (11)*	*1 (1)*
Chronic venous insufficiency thrombosis	*1 (35)*	*1 (23)*	*0 (11)*	*0 (1)*
Treatment				
Spontaneous remission	9 (35)	8 (23)	1 (11)	0 (1)
Dialysis	14 (35)	13 (23)	0 (11)	1 (1)
Plasma therapy	3 (35)	0 (23)	3 (11)	0 (1)
Dialysis and plasma therapy	9 (35)	2 (23)	7 (11)	0 (1)
Eculizumab	5 (35)	2 (23)	3 (11)	0 (1)
Outcome				
Normal renal function	26 (34)	18 (23)	8 (10)	0 (1)
Hypertension[a] and proteinuria[b]	9 (34)	3 (23)	5 (10)	1 (1)
Hypertension[a]	2 (34)	1 (23)	1 (10)	0 (1)
Proteinuria[b]	4 (34)	4 (23)	0 (10)	0 (1)
Maintenance treatment with eculizumab	2 (34)	0 (23)	2 (10)	0 (1)
Relapses after this episode	3 (34)	0 (23)	3 (10)	0 (1)
Genetic or acquired complement aberrations (see Table 9.2)				
Total	15/37 (40.5%)	7/25 (28.0%)	7/11 (63.3%)	1/1 (100%)
CFH	*2/37 (5.4%)*	*2/25 (8.0%)*	-	-
CD46	*2/37 (5.4%)*	-	*2/11 (18.2%)*	-
C3	*3/37 (8.1%)*	*2/25 (8.0%)*	*1/11 (9.1%)*	-
C3 and αFH	*1/37 (2.7%)*	-	*1/11 (9.1%)*	-
αFH	*7/37 (18.9%)*	*3/25 (12.0%)*	*3/11 (27.3%)*	*1/1 (100%)*

For the clinical features, the numbers of patients with data available are reported in parentheses.

[a] Hypertension: a systolic and/ or diastolic pressure ≥ 2,0 Standard Deviation Scores compared to normal values for age, gender and height.

[b] Proteinuria: > 2 y old: > 0,2 mg / mg (> 22,6 mg/ mmol or 0,226 g/10 mmol); < 2 y old: > 0,5 mg/ mg (> 56,6 mg/mmol or 0,566 g/10 mmol).

outcome with normal renal function, although hypertension and/or proteinuria is still present in eight patients (8/23; 34.8%).

Interestingly, more than 70% of the aHUS patients presented with diarrhea and/or gastro-intestinal complaints (e.g. stomach aches, vomiting), but no bloody diarrhea was seen. The other triggering events in aHUS were an upper respiratory tract infection in three patients and headache in one. No extra-renal complications were seen in these patients and the majority of the aHUS patients were treated with both dialysis (average duration: 9 days) and plasmatherapy (average amount of sessions: 14) to control renal failure. Sequelae are still present in 70% of aHUS patients, in most cases both hypertension and proteinuria. For three aHUS patients, the HUS episode at the time of the study was a recurrence. In one of these patients (P20) the first episode was thought to be induced by an STEC infection based on presentation with bloody diarrhea. Two aHUS patients (P6 and P24) were enrolled in the pediatric eculizumab trial.[104] In both, eculizumab treatment had to be discontinued when the trial ended, after which a relapse of disease occurred. These patients are now on maintenance treatment with eculizumab.

Genetic and/or acquired complement aberrations in HUS patients

All but one patient included in the study have been screened for mutations in the complement genes *CFH*, *CFI*, *CD46*, *CFB*, and *C3*, all associated with aHUS, and for the presence of αFH. In 40.5% (15/37) of the patients, including 7/25 STEC-HUS patients and the only SP-HUS patient, we identified a genetic and/or acquired complement abnormality. Characteristics of the identified mutations are depicted in Table 9.2.

Correlation between serological complement profiles and age in healthy children

We included 90 control patients (179 days to 18 years old) to define the correlation between complement levels / activity and age. The serological complement profiles for pediatric controls and the profiles seen in patients in the acute phase of disease are shown in Figure 9.2. No correlation was seen in healthy children between age and serological complement profiles except for C3d levels, which slightly decreased with increasing age (P=0.0017; R^2=0.1086). All levels were within adult reference ranges, although FI levels for pediatric controls are around the lower limit of the adult reference interval. For the comparison of complement profiles between healthy individuals and patients, the results of the pediatric controls were grouped.

Table 9.2. Characteristics of genetic and acquired complement aberrations identified in the enrolled STEC-HUS, SP-HUS, and aHUS patients.

	Disease	Complement aberration	EVS	IHD	Literature
P9	STEC-HUS	αFH	N.A.	N.A.	Dragon-Durey, *et al.*[137] Jozsi *et al.*[82]
P10	STEC-HUS	αFH	N.A.	N.A.	Dragon-Durey, *et al.*[137] Jozsi *et al.*[82]
P14	STEC-HUS	*C3*: p.Arg1219His	1/6503 (0.02%)	0/5036 (0.00%)	
P21	STEC-HUS	*CFH*: p.Thr956Met	15/6503 (0.23%)	1/5036 (0.02%)	Perez-Caballero *et al.*[149] Perkins, Goodship.[239]
P25	STEC-HUS	*C3*: p.Lys155Gln	38/6503 (0.58%)	34/5036 (0.68%)	Seddon *et al.*[184]
P29	STEC-HUS	*CFH*: p.Ser58Ala	2/6503 (0.03%)	0/5036 (0.00%)	
P32	STEC-HUS	αFH	N.A.	N.A.	Dragon-Durey, *et al.*[137] Jozsi *et al.*[82]
P17	SP-HUS	αFH			
P3	aHUS	αFH	N.A.	N.A.	Dragon-Durey, *et al.*[137] Jozsi *et al.*[82]
P8	aHUS	*C3*: p.Arg161Trp; αFH	0/6503 (0.00%)	6/5036 (0.12%)	Roumenina *et al.*[175] Volokhina *et al.*[94]
P18	aHUS	αFH	N.A.	N.A.	Dragon-Durey, *et al.*[137] Jozsi *et al.*[82]
P20	aHUS	*CD46*: p.Asp271_Ser272del	0/6503 (0.00%)	1/5036 (0.02%)	Richards *et al.*[143] Westra *et al.*[72]
P31	aHUS	αFH	N.A.	N.A.	Dragon-Durey, *et al.*[137] Jozsi *et al.*[82]
P33	aHUS	*CD46*: p.Cys35Tyr	0/6503 (0.0%)	2/5036 (0.04%)	Caprioli *et al.*[71]
P35	aHUS	*C3*: p.Arg161Trp	0/6503 (0.00%)	6/5036 (0.12%)	Roumenina *et al.*[175] Volokhina *et al.*[94]

EVS indicates 'Exome variant server (http://evs.gs.washington.edu/EVS/)'; IHD 'in-house database'; N.A. 'not applicable'.

Serological complement profiles in STEC-HUS, aHUS, and SP-HUS patients

Levels of the individual complement proteins C3, FH, and FI, alternative complement pathway activity (AP), and the complement activation products C3d, C3bBbP, C3b/c, and TCC were measured in serum and EDTA plasma of HUS patients on admission and 14-28 days later and were compared to healthy pediatric controls. Levels of the investigated complement proteins in the acute and convalescent phase in all patient groups are shown in Figure 9.3. In the acute phase, patients had slightly decreased complement C3 levels compared to pediatric age-matched controls, although the alterations were not significantly different in all groups. These levels increased in remission in both STEC-HUS and aHUS patients. Levels of the complement regulator Factor I were significantly elevated in both the acute and the convalescent phase of the disease in all patient groups, as compared to the control population (P<0.001); for Factor H levels, this was only the case in STEC-HUS patients (P<0.001 for the acute phase and P<0.01 for the convalescent phase; Figure 9.3).

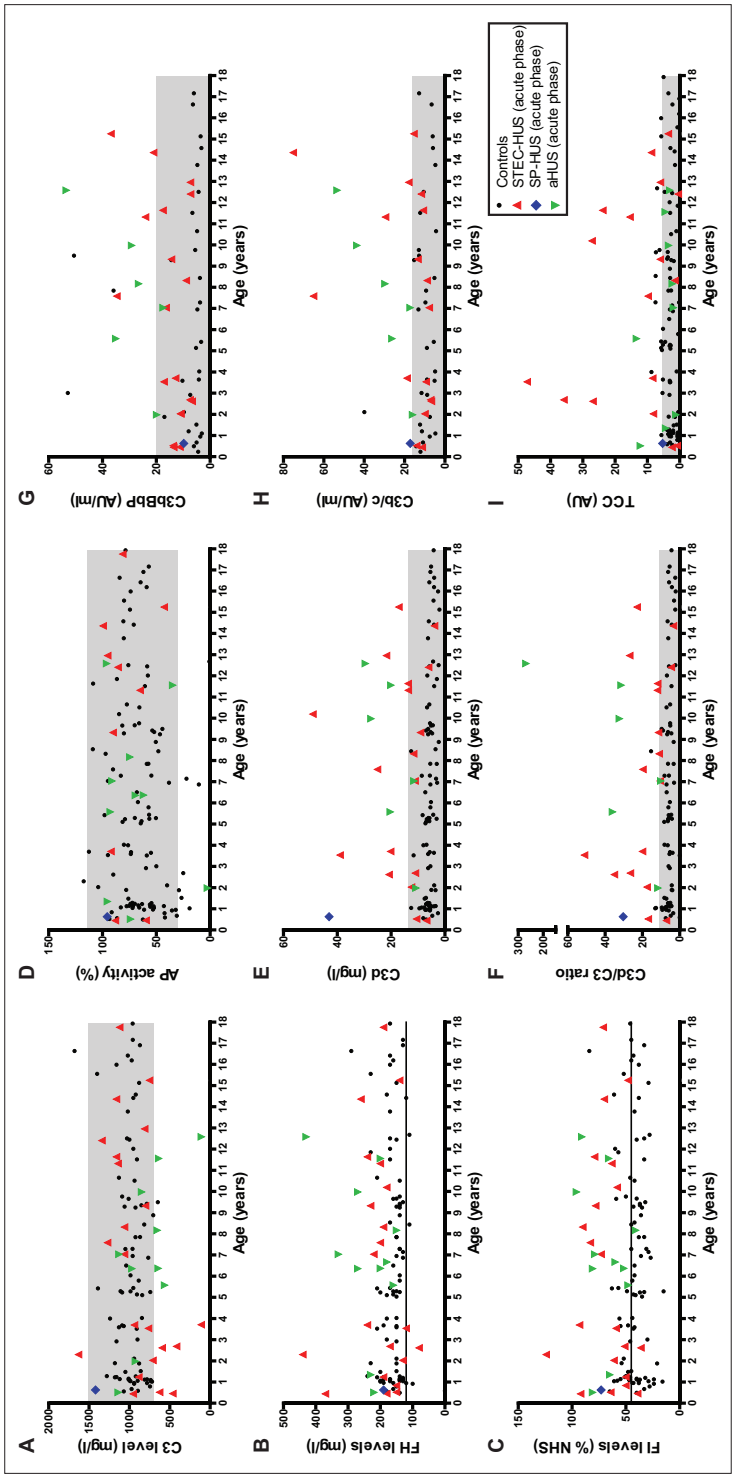

Figure 9.2. Serological complement profiles in controls and patients with STEC-HUS, aHUS, and SP-HUS in the acute phase of disease. Serum and plasma samples of 90 controls (aged 4 months – 18 years) and HUS patients in the acute phase were analyzed for levels of alternative pathway proteins C3, Factor H, and Factor I **(A-C)**, the alternative pathway activity **(D)**, and the complement activation markers C3d, the C3d/C3 ratio, C3bBbP, C3b/c, and TCC **(E-I)**. Each symbol indicates a individual control or patient; controls are symbolized with black dots, STEC-HUS patients with red triangles, aHUS patients with green triangles, and the SP-HUS patients with a blue diamond. Control ranges of adult individuals for C3 (700-1500 mg/l), AP activity (30-113%), C3d (<3.3%), and TCC (<5 AU) levels are shown in grey; reference value for factor H and factor I levels in adults is >120 mg/l and >45%, respectively, indicated with a dotted line. No adult reference ranges are available for the C3d/C3 ratio, C3bBbP and C3b/c, but the reference interval as defined in the present study (mean + two standard deviations of pediatric control values), is depicted with a dotted line. No statistics could be performed for SP-HUS (n=1).

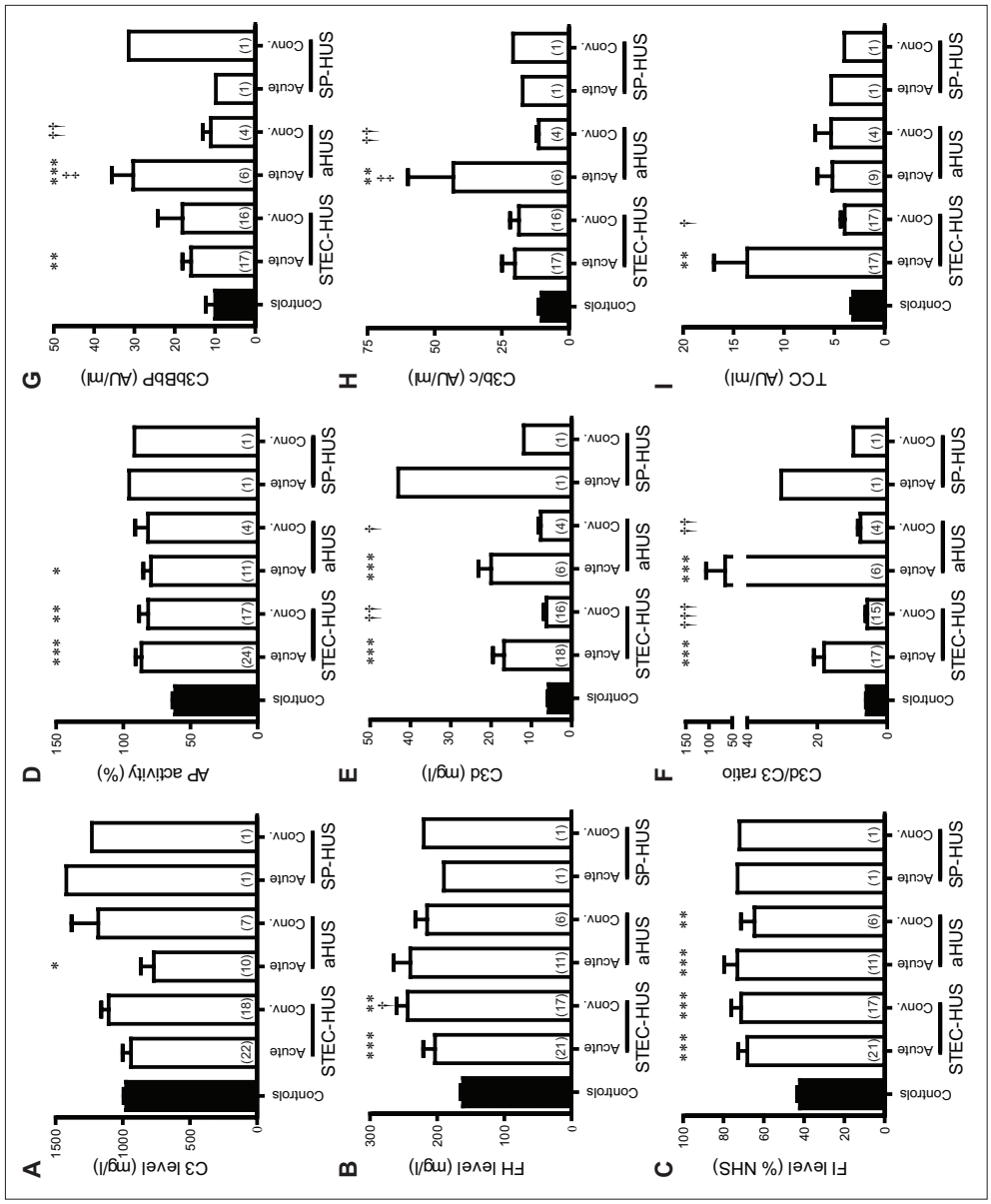

Figure 9.3. Serological levels of individual alternative pathway proteins, alternative pathway activation, and levels of complement activation products in patients with STEC-HUS, aHUS, and SP-HUS in the acute and convalescent phase of disease. Serum and plasma samples of HUS patients in the acute and convalescent phase were analyzed for the complement proteins C3 **(A)**, Factor H **(B)**, and Factor I **(C)**, for alternative pathway activation **(D)**, and for the levels of C3d, C3d/C3 ratio, C3bBbP, C3b/c, and TCC **(E-I)**. The number of screened patients are indicated within parentheses. No statistics could be performed for SP-HUS. Patients vs. controls: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. Convalescent phase vs. acute phase: †, $P<0.05$, ††, $P<0.01$. STEC-HUS vs aHUS: ‡, $P<0.05$.

The mean alternative complement pathway activity in both phases was higher in patients than in controls, but this was not a significant difference for all patient groups (Figure 9.3). Individual AP activity, however, was comparable to respective age-matched controls (Figure 9.2). In the acute phase, the levels of the alternative pathway activation markers C3d (P<0.001 for STEC-HUS and aHUS), C3bBbP (STEC-HUS: P<0.01; aHUS: P<0.001), and C3b/c (STEC-HUS: n.s.; aHUS: P<0.01) were increased as well, as depicted in Figure 9.3. The terminal pathway activation marker TCC was only significantly increased in STEC-HUS patients (P<0.01; Figure 9.3). TCC levels in aHUS and SP-HUS patients, however, were above the mean of pediatric controls as well. In all patient groups, levels of complement activation markers normalized to about control levels in the convalescent phase of disease.

As C3d levels were age-dependent, we calculated the C3d/C3 ratio as an extra marker of alternative complement pathway activation independent of initial C3 levels (Figure 9.3). This ratio did not show an age-dependent effect. In the acute phase of disease, the mean ratio in patients was more than three times higher than in controls (20.00 vs. 5.86), which was statistically significantly different (P=0.0003). No significant difference was seen between STEC-HUS and aHUS patients, but when a cut-off for the C3d/C3 ratio was arbitrarily set at 27.5, based on visual analysis of the data plot, atypical HUS patients were more likely to have a C3d/C3 ratio above this level than STEC-HUS patients (66.7% vs. 11.8%; P=0.021). The other alternative pathway activation markers displayed a significant difference between STEC-HUS and aHUS patients in the acute phase of disease as well (C3bBbP: P=0.011; C3b/c: P=0.027).

The presence of a genetic aberration or αFH could not be linked to an altered serological complement profile: not all patients with a complement aberration displayed complement activation for the analyzed biomarkers in the acute and/or convalescent phase (Supplementary data). However, the power of this analysis was hampered by missing samples for analysis.

Discussion

To investigate the role of the alternative complement pathway in HUS in general (infection-induced HUS and aHUS), samples of 38 children with HUS of any etiology were collected in the acute phase of the disease and 14-28 days afterwards. Levels of individual complement proteins and complement activation markers were measured. It was demonstrated that in the acute phase of disease, the infection-induced HUS and aHUS patients had a trend towards decreased average C3 levels and increased average AP activity, even though this was not statistically different in all patient

groups. The complement activation products C3bBbP, C3b/c, and C3d, and the C3d/C3 ratio were all significantly increased in all patients. This complement activation normalized to control levels in remission. Our results corroborate previous reports of complement activation in children with STEC-HUS and SP-HUS.[65, 91, 110-112, 222]

Thurman *et al*. mention that measurement of TCC might be useful in monitoring the course of the STEC-HUS and recent studies have shown that the activation products C3bBbP, C3b/c, and TCC can be used as biomarker for disease activity in aHUS patients.[237] In this study, the same results were obtained in aHUS patients for C3bBbP and C3b/c, which all normalized in the convalescent phase of disease, but not for TCC. Volokhina *et al*. used remission samples of patients whom had their last aHUS episode more than one year ago, while our samples were already collected within one month after the disappearance of clinical HUS symptoms. It seems that in aHUS, the terminal pathway remains activated for a longer period than the alternative pathway.
No significant difference was seen between acute and convalescent C3bBbP and C3b/c levels in STEC-HUS patients. In all patient groups, the C3d/C3 ratio gives the best discrepancy between the acute phase and the convalescent phase of disease in all patient groups (P<0.01 for STEC-HUS patients and P<0.001 for aHUS patients) and in our experience, the C3d/C3 ratio may therefore be the most promising biomarker to monitor disease activity.

The finding of increased complement activation products in the circulation in the acute phase of aHUS indicate that in this patient group the alternative pathway might not only be dysregulated on the level of the glomerular endothelium, as was indicated in a recent study.[98] Noris *et al.* only measured activation products of the terminal pathway (TCC and C5a), but we and Volokhina *et al*. also investigated the activation products of the alternative pathway.[237] Our results with altered levels of C3bBbP, C3b/c and the C3d/C3 ratio, but not TCC, show that the fluid phase activation most probably occurs mostly at C3 level and not at C5 level.

Based on clinical presentation, it sometimes can be difficult to distinguish between STEC-HUS and aHUS patients: no age differences were seen, many STEC-HUS cases were older than five years, and the majority of aHUS patients (70%) presented with gastro-intestinal problems and/or diarrhea. Not all STEC-HUS patients had (bloody) diarrhea and one aHUS patient presented with diarrhea, which has been described before.[90, 93] This again clearly shows that postdiarrheal onset does not exclude the possibility of aHUS or that the absence of diarrhea excludes STEC-HUS. The C3d/C3 ratio at

admission was on average twice as high in aHUS patients than in infection-induced HUS patients, but this was not a statistically significant difference, most probably due to the low number of patients. At a more detailed inspection of individual ratios, two-third of the aHUS patients had a C3d/C3 ratio over 27.5, while 88% of the STEC-HUS patients had a ratio lower than 27.5. Levels of C3bBbP and C3b/c were also significantly higher in aHUS patient than in STEC-HUS patients. Based on our results, the investigated alternative pathway activation products may therefore be used as a biomarker to discriminate at admission between STEC-HUS and aHUS. As C3d is a more commonly performed measurement than C3bBbP and C3b/c, the C3d/C3 ratio is preferred and this ratio may be used to monitor disease activity as well. As the amount of patients in the current study of examination is very small, a prospective study is needed to determine sensitivity and specificity in a larger cohort before these assays are implemented in routine diagnostics.

As the complement system is one of first parts of the innate immunity to be activated when micro-organisms invade the human body, we cannot exclude that the observed activation in STEC-HUS and SP-HUS is only an infection-related phenomenon, for example due to produced endotoxins by the bacteria. In meningococcal infection, for instance, C3 activation and TCC are both increased with a strong correlation between complement activation and levels of endotoxins produced by *N. meningitides*.[238] A prospective study is therefore needed to investigate differences in complement activation in acute and convalescent phase between patients that develop HUS after an infection with STEC or SP, those that do not, and patients with other severe life-threatening infections.

We were surprised to see that in almost 30% of the STEC-HUS patients and in the SP-HUS patient a genetic complement aberration could be identified. Several case reports of STEC-HUS patients with complement mutations have been published, but so far no large cohorts were investigated.[48, 50-52] Most of the identified genetic variations were described before in other diseases associated with a dysregulated complement system (aHUS and/or age-related macular degeneration [AMD]) and functional studies that have been performed showed that these aberrations influence binding capacity of complement to C3b regulators or affect the inactivation of C3.[71, 72, 94, 143, 180, 184, 239] All genetic aberrations we have identified were present in the EVS database and/or in the in-house database with exome data of >5000 individuals (Table 9.2). This is not remarkable, as an incomplete penetrance is seen in HUS: healthy family members can carry disease-causing mutations.[29, 92] This indicates that additional triggers, genetic and/or environmental, are probably needed for the disease to develop.

As in other studies, we could not link an altered complement profile in both the acute and convalescent phase of the disease to the presence of a genetic or acquired complement aberration (mutation or αFH).[98] Both patients with and without identified mutations displayed similar complement abnormalities on protein level and not all patients with a complement aberration demonstrated complement activation for the analyzed biomarkers in the acute phase and/or in remission.

The finding of mutations and αFH in STEC and SP-patients argues that there might be undiagnosed cases of aHUS triggered by an STEC or SP infection on a genetic background of impaired complement regulation. Different than in aHUS patients, the so-far investigated infection-induced HUS patients of our study with complement mutations or αFH had a good outcome and no recurrences. These patients, however, might be prone to develop a recurrence of disease. Indeed, one of the aHUS patients (P20) had a previous episode of HUS preceded by bloody diarrhea, but the recurrent episode had no evidence of a preceding STEC infection. In this patient, a *CD46* mutation was identified (Table 9.2). The same occurred in two Italian patients: they were first diagnosed with STEC-HUS, but after an HUS recurrence occurred, a complement mutation was identified.[90]

For the investigated complement protein levels or activation products, except for C3d, no age-dependent effect was seen in the control individuals we included in this study (age >5 months). It has been shown in neonatal infants at birth that complement levels are lower than in adults and rise towards adult levels within approximately 6 months.[224, 228] To investigate the maturation of the alternative complement pathway more extensively, a new study to extend the collection of pediatric controls via a strict protocolled manner with children younger than 6 months is ungoing.

Together, we conclude that in both infection-induced HUS and aHUS patients the complement system is activated in the fluid phase in the acute phase of disease, but not in remission. The C3d/C3 ratio seems to display the best discrepancy between STEC-HUS and aHUS and between the acute and convalescent phase and may therefore be used as a biomarker for disease activity, but a larger cohort is needed to determine sensitivity and specificity of the assay. A genetic or acquired complement aberration was present in 40.5% of the patients, including STEC-HUS and SP-HUS patients, but both patients with and without complement aberrations showed altered complement profiles to a variable extent. As the complement system is the first part of the innate immune system that is activated after infection, we cannot exclude that the activation is purely infection-

related. A international prospective study to investigate the role of the complement system, including the presence of complement mutations and/or autoantibodies against complement proteins, in a larger cohort with patients that do and do not develop HUS after infection is needed.

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Supplementary data.

Serological and genetic complement profiles of HUS patients in the acute and convalescent phase of disease. Levels outside the control ranges for pediatric controls (defined by this study as mean ± 2 standard deviations) are shown in bold and indicated with (↑) or (↓). Patient 24 was on eculizumab in both convalescent phases; patient 8 and 24 had a second episode of aHUS.

Patient	Sex	Age	Disease	Serotype	Acute phase									Convalescent phase								Genetics	αFH	αO157-LPS
					AP (%NHS)	C3 (mg/l)	FH (mg/l)	FI (%NHS)	C3d (mg/l)	C3d/C3 ratio	C3bBbP (AU/ml)	C3b/c (AU/ml)	TCC Hycult	AP (%NHS)	C3 (mg/l)	FH (mg/l)	FI (%NHS)	C3d (mg/l)	C3d/C3 ratio	C3bBbP (AU/ml)	C3b/c (AU/ml)	TCC Hycult			
P1	M	2y 7m	STEC-HUS	O26	90,1	**594 (↓)**	**80 (↓)**	36	**20,78 (↑)**	**34,99 (↑)**	6,67	**17,62 (↑)**	**26,84 (↑)**	106,7	**1320 (↑)**	280	75	**15,86 (↑)**	**12,02 (↑)**	11,66	**38,31 (↑)**	5,23	no aberrations	NEG	NEG
P4	F	10y 2m	STEC-HUS	O157	84,7	N/D	180	58	**49,07 (↑)**	N/D	N/D	N/D	**27,14 (↑)**	94,6	N/D	110	30	N/D	N/D	**28,91 (↑)**	**49,69 (↑)**	4,43	no aberrations	NEG	**POS**
P5	M	3y 8m	STEC-HUS	O26	112,7	937	240	93	N/D	N/D	N/D	N/D	N/D	**120,0 (↑)**	**1440 (↑)**	320	90	7,67	5,33	6,22	14,01	4,43	no aberrations	NEG	NEG
P7	F	2y 3m	STEC-HUS	O157	**117,5 (↑)**	**1630 (↑)**	440	124	N/D	N/D	N/D	N/D	N/D	82,1	1140	270	75	6,89	6,05	14,03	**20,94 (↑)**	3,43	no aberrations	NEG	NEG
P9	F	0y 10m	STEC-HUS	O104	91,5	N/D	150	50	N/D	N/D	N/D	N/D	N/D	89,5	**1560 (↑)**	280	79	8,01	5,13	15,22	**28,86 (↑)**	5,98	no aberrations	**POS**	NEG
P10	M	11y 7m	STEC-HUS	O157	**108,8 (↑)**	1160	240	79	**13,69 (↑)**	**11,81 (↑)**	17,54	10,73	**23,82 (↑)**	103,3	1180	190	69	4,70	3,98	7,29	6,03	3,99	*no DNA available*	**POS**	**POS**
P12	M	8y 6m	STEC-HUS	O157	95,1	1060	220	73	**11,15 (↑)**	10,52	16,39	7,74	**9,76 (↑)**	69,9	930	180	75	4,13	4,44	**106,88 (↑)**	**38,27 (↑)**	5,68	no aberrations	NEG	**POS**
P13	M	7y 7m	STEC-HUS	O157	90,3	1270	200	83	**25,08 (↑)**	**19,75 (↑)**	**34,69 (↑)**	**65,09 (↑)**	2,48	*No material available in convalescent phase of disease*									no aberrations	NEG	**POS**
P14	F	2y 8m	STEC-HUS	O5	25,1	**415 (↓)**	170	51	**10,98 (↑)**	**26,45(↑)**	7,48	7,02	**35,85 (↑)**	103,4	N/D	360	99	3,68	N/D	10,25	10,11	4,77	**C3: p.Arg1219His**	NEG	NEG
P15	M	3y 6m	STEC-HUS	O157	95,1	764	120	59	**38,96 (↑)**	**50,99 (↑)**	17,12	9,33	**47,22 (↑)**	**114,5 (↑)**	1270	290	101	5,59	4,40	15,72	10,32	4,97	no aberrations	NEG	NEG
P16	F	2y 0m	STEC-HUS	NT	**104 (↑)**	709	130	61	**12,42 (↑)**	**17,52 (↑)**	10,97	9,89	**8,14 (↑)**	102,1	928	190	59	**12,42 (↑)**	N/D	**21,14 (↑)**	**16,15 (↑)**	3,29	no aberrations	NEG	NEG
P19	F	8y 4m	STEC-HUS	NT	97,2	1060	190	90	**11,65 (↑)**	**10,99 (↑)**	8,85	8,76	1,48	*No material available in convalescent phase of disease*									no aberrations	NEG	NEG
P21	F	6y 3m	STEC-HUS	O157	*No material available in acute phase of disease*									N/D	1230	240	97	7,11	5,78	N/D	N/D	0,11	**CFH: p.Thr956Met**	NEG	**POS**
P22	M	0y 6m	STEC-HUS	O157	93,7	**626 (↓)**	150	64	**10,54 (↑)**	**16,84 (↑)**	13,73	13,94	0,77	*No material available in convalescent phase of disease*									no aberrations	NEG	**POS**
P23	M	1y 3m	STEC-HUS	O157	74,1	884	190	50	N/D	N/D	N/D	N/D	N/D	*No material available in convalescent phase of disease*									no aberrations	NEG	**POS**
P25	F	2y 0m	STEC-HUS	O157	*No material available in acute phase of disease*									**8,1**	1310	340	81	6,44	4,91	10,87	9,27	4,79	**C3: p.Lys155Gln**	NEG	**POS**
P26	M	11y 4m	STEC-HUS	NT	65,0	1140	200	63	**13,61 (↑)**	**11,93 (↑)**	**24,00 (↑)**	**29,45 (↑)**	**15,38 (↑)**	77,5	1040	220	39	4,14	3,98	4,89	5,38	6,47	no aberrations	NEG	NEG
P27	F	0y 5m	STEC-HUS	NT	88,0	953	370	92	6,78	N/D	13,52	11,21	2,40	66,3	710	140	63	N/D	N/D	N/D	N/D	N/D	no aberrations	NEG	NEG
P28	M	0y 5m	STEC-HUS	O26	59,5	462	180	39	N/D	N/D	N/D	N/D	N/D	67,6	813	250	48	7,59	N/D	11,21	**18,67 (↑)**	0,49	no aberrations	NEG	NEG
P29	F	9y 4m	STEC-HUS	O26	90,2	801	230	78	9,09	N/D	14,36	13,39	6,02	42,6	913	230	65	4,64	5,09	6,88	9,96	2,97	**CFH: p.Ser58Ala**	NEG	NEG
P30	F	15y 3m	STEC-HUS	O157	42,7	750	140	48	**17,10 (↑)**	N/D	**36,89 (↑)**	15,36	3,53	70,8	1030	260	67	3,91	N/D	11,35	12,46	2,35	no aberrations	NEG	**POS**
P32	M	17y 9m	STEC-HUS	O157	81,2	1120	190	71	N/D	N/D	N/D	N/D	N/D	*No material available in convalescent phase of disease*									no aberrations	**POS**	**POS**
P33	M	6y 4m	STEC-HUS	NT	62,1	645	200	52	N/D	N/D	N/D	N/D	N/D	*No material available in convalescent phase of disease*									**CD46: p.Cys35Tyr**	NEG	NEG
				Control range	24-99 %NHS	639-1312 mg/l	>97 mg/l	>19,5 %NHS	<9,73 mg/l	<10,82	<19,88 AU/ml	<15,92 AU/ml	<7,0 AU/ml	24-99 %NHS	639-1312 mg/l	>97 mg/l	>19,5 %NHS	<9,73 mg/l	<10,82	<19,88 AU/ml	<15,92 AU/ml	<7,0 AU/ml			

N/D indicates 'not determined'; N/A 'not applicable.

Patient	Sex	Age	Disease	Serotype	Acute phase									Convalescent phase									Genetics	αFH	αO157-LPS
					AP (%NHS)	C3 (mg/l)	FH (mg/l)	FI (%NHS)	C3d (mg/l)	C3d/C3 ratio	C3bBbP (AU/ml)	C3b/c (AU/ml)	TCC Hycult	AP (%NHS)	C3 (mg/l)	FH (mg/l)	FI (%NHS)	C3d (mg/l)	C3d/C3 ratio	C3bBbP (AU/ml)	C3b/c (AU/ml)	TCC Hycult			
P34	M	14y 4m	STEC-HUS	O157	**99,4 (↑)**	1160	260	70	3,87	N/D	**21,03 (↑)**	**75,15 (↑)**	**8,72 (↑)**	*No material available in convalescent phase of disease*									no aberrations	NEG	**POS**
P36	F	12y 11m	STEC-HUS	O157	95,2	810	N/D	N/D	**21,72 (↑)**	**26,82 (↑)**	7,34	**17,91 (↑)**	5,97	N/D	950	N/D	N/D	N/D	N/D	N/D	N/D	N/D	no aberrations	NEG	**POS**
P37	F	12y 5m	STEC-HUS	O157	85,5	1340	N/D	N/D	6,07	N/D	7,29	11,70	0,44	N/D	1280	N/D	N/D	N/D	N/D	N/D	N/D	N/D	no aberrations	NEG	**POS**
P38	M	3y 8m	STEC-HUS	O157	92,2	1010	N/D	N/D	**20,15 (↑)**	**19,95 (↑)**	12,83	**18,77 (↑)**	**8,35 (↑)**	67,0	864	N/D	N/D	4,18	N/D	7,41	10,49	3,29	no aberrations	NEG	**POS**
P17	F	0y 7m	SP-HUS	-	95,7	**1420 (↑)**	190	73	**43,05 (↑)**	**30,32 (↑)**	9,81	**17,26 (↑)**	5,31	91,8	1230	220	72	**11,90 (↑)**	9,68	**31,45 (↑)**	**20,87 (↑)**	4,01	no aberrations	**POS**	NEG
P2	M	4y 2m	aHUS	-	95,6	N/D	230	65	N/D	N/D	N/D	N/D	4,38	101,1	2170	270	88	N/D	N/D	N/D	N/D	N/D	no aberrations	NEG	NEG
P3	M	6y 8m	aHUS	-	69,5	N/D	180	60	N/D	N/D	N/D	N/D	N/D	N/D	1520	150	53	N/D	N/D	N/D	N/D	N/D	no aberrations	**POS**	NEG
P6	F	6y 4m	aHUS	-	91,1	970	270	81	N/D	N/D	N/D	N/D	N/D	*No material available in convalescent phase of disease*									no aberrations	NEG	NEG
P8	M	12y 7m	aHUS	-	96,3	**110 (↓)**	430	91	**29,64 (↑)**	**269,49 (↑)**	**53,51 (↑)**	**125,24 (↑)**	3,11	*No material available in convalescent phase of disease*									**C3: p.Arg161Trp**	NEG	NEG
P11	F	7y 0m	aHUS	-	91,3	1130	330	115	**11,56 (↑)**	10,23	17,51	**17,27 (↑)**	2,00	*No material available in convalescent phase of disease*									no aberrations	NEG	NEG
P18	F	11y 6m	aHUS	-	34,9	639	200	66	**20,13 (↑)**	**31,50 (↑)**	N/D	N/D	4,52	94,6	1190	240	58	6,40	5,38	7,39	10,02	5,58	no aberrations	**POS**	NEG
P20	M	8y 2m	aHUS	-	74,7	657	150	41	N/D	N/D	**26,60 (↑)**	**29,76 (↑)**	2,35	*No material available in convalescent phase of disease*									**CD46: p.Asp271_Ser272del**	NEG	NEG
P24	M	0y 6m	aHUS	-	74,1	1140	220	81	N/D	N/D	N/D	N/D	**12,24 (↑)**	N/D	1000	220	81	8,28	8,28	14,07	11,4	**9,39 (↑)**	no aberrations	NEG	NEG
		1y 11m	aHUS	-	91,2	927	N/D	N/D	**10,84 (↑)**	**11,69 (↑)**	**19,93 (↑)**	**16,09 (↑)**	1,21	N/D	899	N/D	N/D	7,39	7,39	14,75	10,48	4,52	no aberrations	NEG	NEG
P31	M	5y 7m	aHUS	-	**2,7 (↓)**	**620 (↓)**	160	56	**20,39 (↑)**	**32,88 (↑)**	**35,12 (↑)**	**26,33 (↑)**	**13,43 (↑)**	64,0	**530 (↓)**	180	46	N/D	N/D	N/D	N/D	N/D	no aberrations	**POS**	NEG
P35	F	9y 11m	aHUS	-	92,9	850	270	96	**27,53 (↑)**	**32,38(↑)**	**29,20 (↑)**	**43,70 (↑)**	3,46	67,2	970	230	62	8,73	N/D	N/D	N/D	1,78	**C3: p.Arg161Trp**	NEG	NEG
				Control range	24-99 %NHS	639-1312 mg/l	>97 mg/l	>19,5 %NHS	<9,73 mg/l	<10,82	<19,88 AU/ml	<15,92 AU/ml	<7,0 AU/ml	24-99 %NHS	639-1312 mg/l	>97 mg/l	>19,5 %NHS	<9,73 mg/l	<10,82	<19,88 AU/ml	<15,92 AU/ml	<7,0 AU/ml			

N/D indicates 'not determined'; N/A 'not applicable.

Chapter 10

Identification and functional characterization of potential complement evasion factors secreted by STEC O157:H7

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In preparation.

Abstract

Hemolytic uremic syndrome (HUS) is one of the major causes of renal failure in childhood. Most cases of the disease are caused by infection with Shiga-toxin producing *Escherichia coli* (STEC). Available experimental evidence suggests that complement activation may play an important role in STEC-HUS pathogenesis, however the exact etiology of this disease is not yet fully understood.

In this study, we used *in silico* genome analysis to select STEC-specific genes encoding secreted proteins that are not present in non-STEC *E. coli* genomes. In total, ten proteins were selected, of which five were successfully produced with a His-tag using recombinant technology and purified using TALON metal affinity resin. Complement activation was induced in normal human serum in the presence or absence of the STEC proteins. Binding to complement proteins and resulting complement activation was assessed by measuring C3b and TCC (C5b-9 and C6-9) levels using ELISA. None of the selected proteins were able to alter complement activation in human serum.

These results indicate that a more robust approach is needed to analyze complement dysregulating properties of the STEC secretome, such as studies using the phage display library, which are currently being performed.

Introduction

The hemolytic uremic syndrome (HUS) is one of the major causes of acute renal failure in children. It is characterized by hemolytic anemia, thrombocytopenia and acute renal failure. More than 90% of cases (STEC-HUS) are preceded by watery or bloody diarrhea that are caused by an infection with Shiga toxin-producing *Escherichia coli* (STEC), which can be ingested with improperly cooked meat or contaminated water and vegetables.[37] A STEC infection leads to HUS in approximately 15% of cases.[35] The majority of STEC-HUS cases affects children and is associated with infection by the O157:H7 STEC serotype. In the intestine, HUS-causing STEC-strains produce Shiga toxins belonging to type Stx1 and/or type Stx2.[240, 241]

Although Stx1 and Stx2 have been linked to protein synthesis inhibition, triggering of apoptosis, complement activation, and regulation of gene expression of cytokines and chemokines, the exact mechanisms of how Stx exposure leads to HUS is still not completely understood.[242]

The complement system plays an important role in the innate immunity. It can be activated via three pathways: the classical, the lectin and the alternative.[66, 210] The alternative complement pathway dysregulation, which is well-characterized in non-STEC-HUS[3, 29, 158], may be important in STEC-HUS as well. Already in the 1980's, increased levels of the breakdown products of C3 and complement factor B (FB) were demonstrated by our group in serum samples of children with diarrhea-associated HUS.[65] In addition, a more recent study reported increased serum levels of Bb and serum C5b-9 in children in the acute phase of STEC-HUS.[111] Complement activation was also shown on platelet-leukocyte complexes and microparticles of HUS patients[112]. This complement activation in STEC-HUS might partially be attributed to Shiga toxin. *In vitro* Stx2 binds complement factor H (FH) and is able to activate alternative complement pathway.[113] Furthermore, *in vitro* Stx1 triggers complement C3 activation at the cell surface, which is mediated by P-selectin.[116] Manipulation of complement activation is not reserved for the Shiga toxins. The STEC autotransporter *E. coli* secreted protein P (EspP) was shown to be able to cleave C3/C3b and C5 and impair complement activation in serum.[117] Furthermore, the zinc metalloprotease StcE found in STEC O157:H7 is able to downregulate the classical complement pathway (reviewed in S. Rooijakkers et al. [118]). These examples illustrate that STEC is very well able to manipulate complement in multiple ways, as are other bacteria like *Staphyloccus aureus*, *Borrelia Burgdorferi*, and several streptococci.[114, 118]

In this project, we aim to discover and functionally characterize novel complement evasion factors produced by STEC via well-established methods[118], which might lead to the development of the new treatment strategies to protect renal function in the STEC-HUS patients in the future.

Materials and Methods

Bacterial strains and growth conditions

E. coli strains O157:H7 Sakai, DH5α (laboratory stock), and BL21(DE3) (Novagen) were grown at 37°C or 25°C in Luria-Bertani (LB) medium or on LB agar plates, supplemented, when required, with 100 µg/ml of ampicillin (Sigma-Aldrich).

Plasmid constructions

DNA fragments, containing mature STEC proteins were obtained by PCR from genomic DNA of the O157:H7 Sakai strain (primers available upon request). The PCR products were cloned into the modified pET302/NT-His expression vectors (Invitrogen) using *Bam*H I and *Not* I expression sites. In case of the *ECs4562* gene, which contains an internal *Bam*H I site, *Bgl* II was used. Combinations of different primers and vectors allowed to obtain constructs encoding various 6xHis-tagged variants as well as tag-free proteins (Figure 10.1).

Expression of recombinant proteins

Strain BL21(DE3) containing the obtained expression vectors was used to produce proteins. The BL21(DE3) cells were grown in 300 ml of total culture volume. When an optical density at 600 nm reached 0.7 OD, 100 µM of isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce the expression of the recombinant proteins. After 90 min of induction at 37°C (ECs2714 N-His FXa) or overnight induction at 25°C (all other preparations), the cells were harvested by centrifugation and frozen at –20°C.

Protein isolation

The BL21(DE3) bacteria with the expression vectors were subjected to osmotic shock in order to remove periplasmic fraction containing metal chelators.[243] To this end, the cell pellets were resuspended in sucrose buffer (50 mM HEPES, 20% sucrose, 1mM EDTA, pH 7.9) at 5 ml/g and centrifuged (2100 g, 30 min, 4°C). The supernatant was removed and the pellet was resuspended in 5 mM $MgSO_4$ at 5 mL/g and incubated on ice for 10 min. The pellet was collected by centrifugation (2100 g, 30 min, 4°C) and stored at -20°C.

Pellets obtained after the osmotic shock procedure were lysed in 45 ml CelLytic™ B (Sigma-Aldrich), supplemented with 1 tablet of EDTA-free protease inhibitor cocktail, 28 µg/ml of DNase I and RNase A (all from Roche diagnostics) and 100 µg/ml lysozyme (Sigma-Aldrich) and incubated for 30 min on

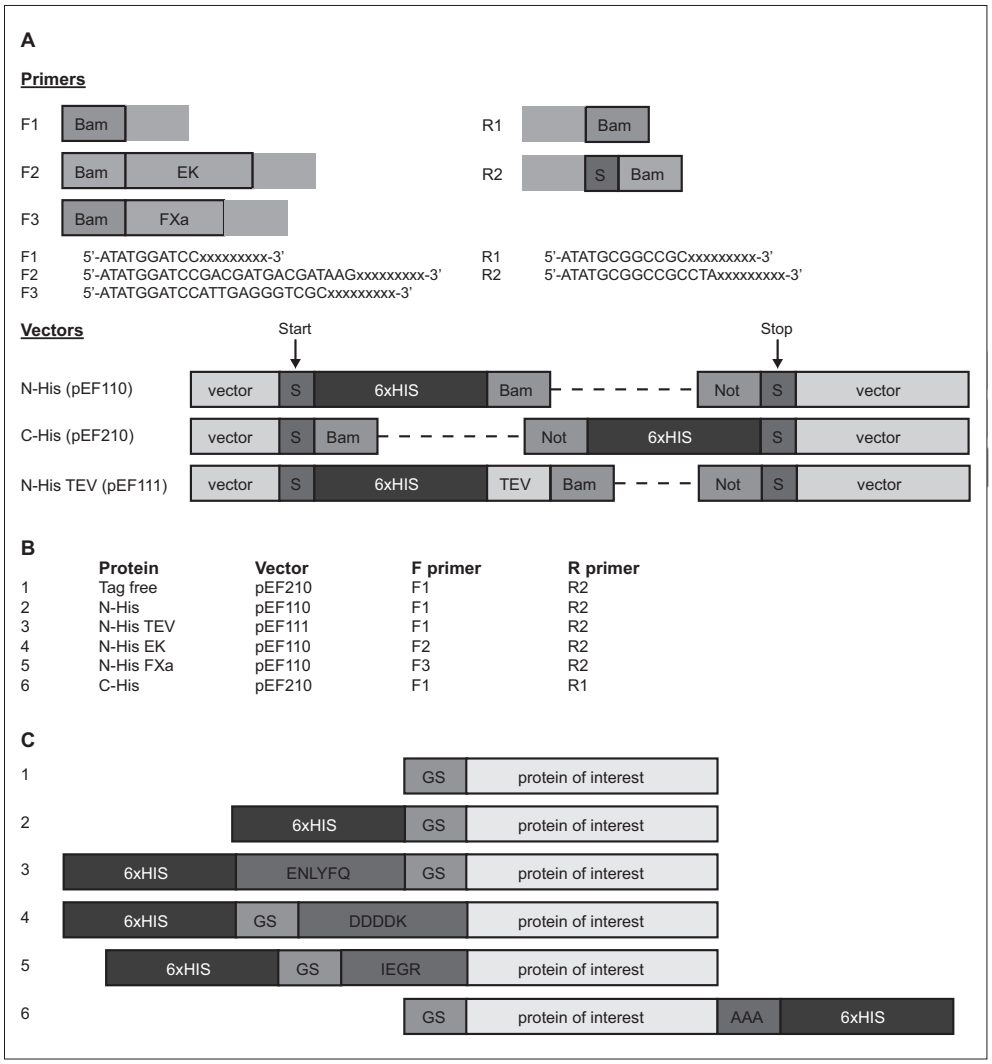

Figure 10.1. Cloning strategy for the recombinant production of proteins. (A) Schematic representation of the five primers: three forward (F1-F3) and two reverse (R1, R2); and three vectors: pEF110, pEF210 and pEF 111, used in this study are given. Locations of the *Bam*H I (Bam) and *Not* I (Not) sites are shown. The N-terminal (N-His) and C-terminal (C-His) positions of the sequences encoding polyhistidine tag (6xHIS) and start and stop codons (S) in the vectors are indicated. Sequences encoding cleavage sites for the removal of the polyhistidine tag specific for enterokinase (EK), factor Xa (FXa), and tobacco etch virus (TEV) protease are specified. **(B)** Combinations of the specific vectors and primers, required for the production of the six possible protein variants. **(C)** Schematic representation of the resulting six protein variants, including polyhistidine tags, protease cleavage sites, and additional amino acids (GS) encoded by the *Bam*H I site that were introduced in the cloning process.

a shaker at room temperature. The inclusion bodies were collected by centrifugation (47800 g, 20 min, 4°C); washed twice by resuspending in CelLytic^{TM} B diluted 1:10 in H_20, and centrifuging (47800 g, 20 min, 4°C); and solubilized in denaturing buffer (8 M urea, 20 mM sodium phosphate buffer, 0.5 M NaCl, pH 8.5). The remaining insoluble material was removed by centrifugation (47800 g, 20 min, 4°C).

Affinity purification and refolding

The obtained preparations of the solubilized His-tagged proteins were purified using TALON metal affinity resin (Clontech) according to the manufacturer's protocol for denaturing conditions. The proteins were eluted in elution buffer (8 M urea, 20 mM sodium phosphate buffer, 0.5 M NaCl, 300 mM imidazole, pH 7.8).

The purified proteins were dialyzed against the refolding buffer (50 mM Tris, 0.5 M L-argenine, pH 10), and after that dialyzed against phosphate buffered saline (PBS; pH 8) for complement assays. Protein preparations were analyzed using SDS-PAGE and Coomassie Brilliant Blue staining.

Complement assays

Complement activation of classical and alternative pathways was analyzed separately. Normal human serum (NHS) was diluted to create a concentration ranges of 0, 2.5, 5 and 10 % (classical pathway assay) and 0, 5, 25 and 50 % (alternative pathway assay) prior to complement activation. Generation of TCC reflected by C5b-9 and C6-9 levels, and of C3b was quantified in ELISA setting.[210]

Binding of the purified proteins to complement factors was analyzed in ELISA setting. To this end, FH, FI, FD, FB, and C3b were coated overnight at 4°C on a 96-wells plate at concentrations of 1, 5 and 10 µg/ml in carbonate buffer (15 mM Na_2CO_3, 25 mM $NaHCO_3$, pH 9.6), and incubated with STEC proteins C diluted at a concentration of 10 µg/ml in dilution buffer (PBS, 1% bovine serum albumin, 0.05% tween-20) for 60 min. at 37°. Immunodetection was performed using a primary mouse anti-His antibody (Invitrogen) and a secondary horseradish peroxidase-labeled goat-anti-mouse antibody (Dako).

Results

Identification of possible complement evasion factors

Comparative analysis of STEC and non-STEC *E. coli* genomes, was performed using the total genome and sequence analysis software Kodon (Applied Maths). In total 21 non-STEC and nine STEC

genomes were compared (Table 10.1); only two of these STEC strains have directly been involved in an HUS outbreak (Sakai and TW14359). The ten proteins that were STEC-specific, annotated as secreted, had a low molecular weight (<25 kDa) and a high isoelectric point (>8), are secreted via the type 3 secretion system of STEC, and were encoded by genes that are located on mobile elements, were chosen for further production and analysis (Table 10.2). All selected genes are present in O157:H7 Sakai strain, a well-described HUS-causing STEC strain, and.[244, 245]

Table 10.1. *Escherichia coli* genomes used in comparative analysis.

None STEC-strains	STEC strains
Escherichia coli 'BL21-Gold(DE3)pLysS AG'	*Escherichia coli* O103:H2 str. 12009
Escherichia coli 536	*Escherichia coli* O111:H- str. 11128
Escherichia coli 55989	*Escherichia coli* O127:H6 str. E2348/69
Escherichia coli APEC O1	*Escherichia coli* O157:H7 EDL933
Escherichia coli ATCC 8739	*Escherichia coli* O157:H7 str. EC4115
Escherichia coli B str. REL606	*Escherichia coli* O157:H7 str. Sakai
Escherichia coli BW2952	*Escherichia coli* O157H7 str. TW14359
Escherichia coli CFT073	*Escherichia coli* O26:H11 str. 11368
Escherichia coli E24377A	*Escherichia coli* O55:H7 str. CB9615
Escherichia coli ED1a	
Escherichia coli HS	
Escherichia coli IAI1	
Escherichia coli IAI39	
Escherichia coli S88	
Escherichia coli SE11	
Escherichia coli SMS-3-5	
Escherichia coli UMN026	
Escherichia coli UTI89	
Escherichia coli str. K-12 substr. DH10B	
Escherichia coli str. K-12 substr. MG1655	
Escherichia coli str. K-12 substr. W3110	

Table 10.2. Possible novel complement evasion factors of the *E. coli* strain O157:H7 Sakai, selected by comparative genome analysis. The isoelectric point (pI) and molecular weight (Mw) values are given for the tag-free wild type variants. ECs2715 and ECs1126 are also known as TccP and TccP2, respectively.[246, 252]

	pI	MW (kDa)
ECs1825 (EspM1)	9.1	21.9
ECs3485 (EspM2)	8.7	22.5
ECs4562 (Map)	9.7	22.9
ECs2714 (EspJ)	9.5	24.7
ECs4550 (EspF1)	10.4	25.3
ECs2715 (EspF2-2)	10.1	37.2
ECs1126 (EspF2-1')	6.4	21 .0
ECs4564 (EspH)	9.8	19.3
ECs1567 (EspO1-1)	9.1	10.3
ECs1821 (EspO1-2)	8.6	10.4

Production and purification of recombinant protein

Potential STEC complement evasion factors were produced in recombinant form with a His-tag and purified using affinity chromatography, as described in Materials and Methods. For one gene (*ECs1126*), it was not possible to obtain a PCR product due to high frequency of short homologues repeats in the locus of the gene.

To optimize the yields of protein expression, IPTG concentrations (50, 100, 300 µM) and expression temperature and time (37˚C for 90 min or 25˚C overnight) were varied. Furthermore, the genes were cloned as tag-free variants and with various His-tag constructs: a N-terminal His-tag (cleavable and not cleavable) and a C-terminal His-tag (Figure 10.1). The best obtained preparations are illustrated in Figure 10.2. For the proteins ECs1825, ECs4564, ECs1567 and ECs1821 only low yields of expression were obtained despite the thorough optimization attempts.

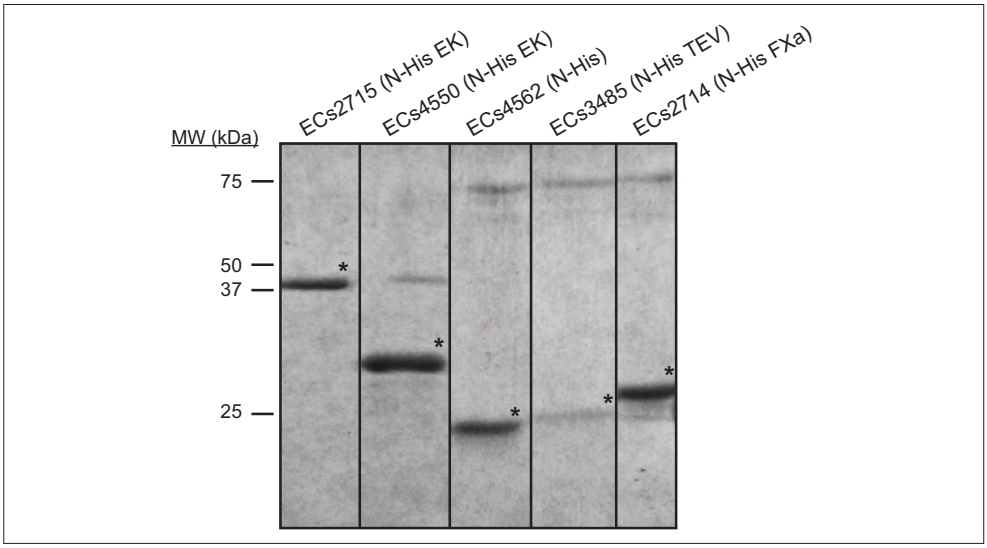

Figure 10.2. The obtained final preparations of the five recombinant STEC proteins. Samples were analyzed using SDS-PAGE and Coomassie Brilliant Blue staining. A molecular weight standard is indicated on the left. Bands corresponding to the molecular weights of proteins are indicated (*).

Functional analysis of possible complement evasion factors

Next we analyzed possible effect of produced recombinant STEC proteins on complement activation in the fluid phase. Proteins ECs3485, ECs4562, ECs2714, ECs4550 and ECs2715 yielded enough amount for these experiments (Figure 10.2, Figure 10.3). None of the proteins affected levels of complement activation in NHS. The ECs4550 protein appeared to increase efficiency of C5b-9 generation in alternative complement pathway activation in one of the experiments (Figure 10.3A).

However, this finding was not supported by the measurements of C3b and C6-9 levels (Figure 10.3B and 10.3C).

Moreover, we performed binding studies to analyze possible interactions between the STEC proteins and human complement proteins (FH, FI, FD, FB, and C3b). For these assays amounts obtained for all nine produced STEC proteins were sufficient. No binding between complement factors and the produced STEC proteins was observed in these experiments (data not shown).

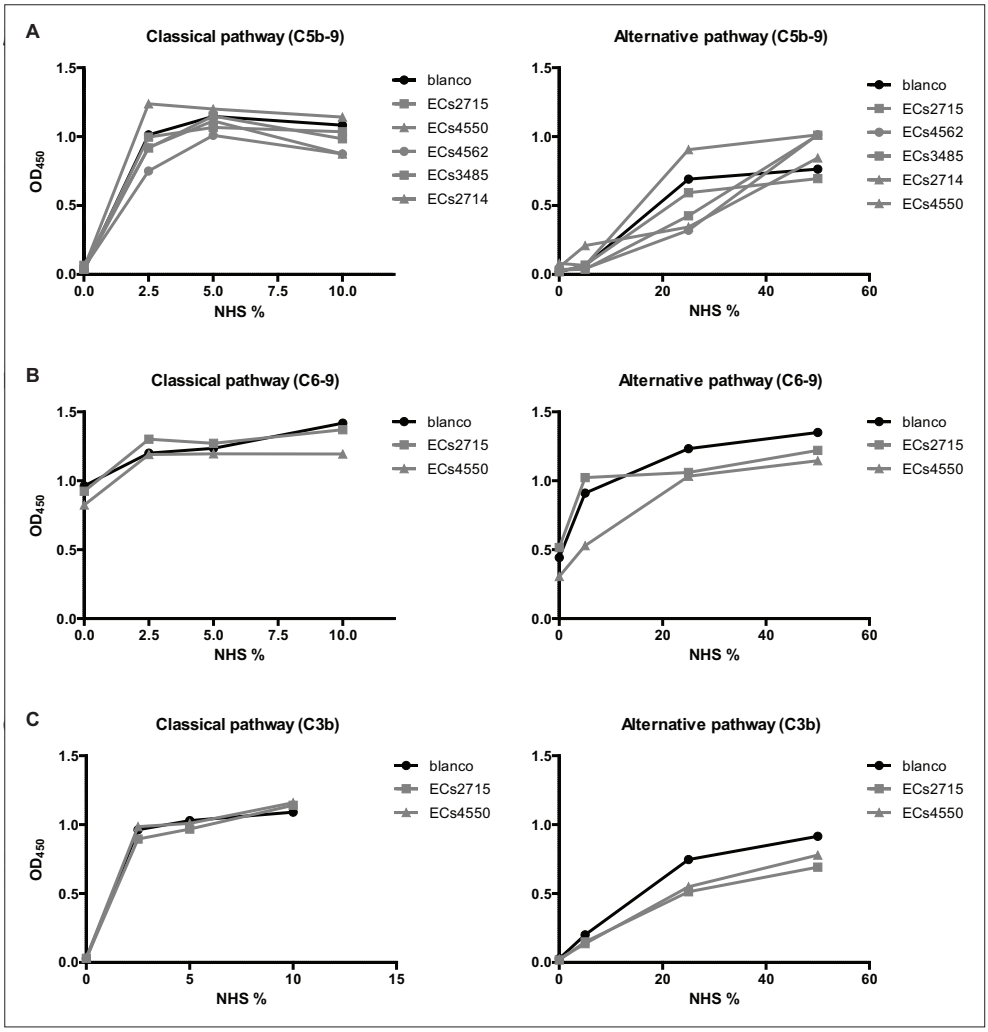

Figure 10.3. Results of the complement activation experiments. Complement activation was induced in diluted NHS in the absence (blanco sample) or presence of recombinant STEC proteins. Samples were collected and complement activation was assessed by measuring levels of C5b-9 **(A)**, C6-9 **(B)** and C3b **(C)**, generated during specific activation of the classical and alternative pathways. C5b-9 and C6-9 both reflect levels of the terminal complement component (TCC).

Discussion

In the light of the complement evading properties of other bacteria, we aimed to discover and characterize novel evasion factors produced by STEC, next to Stx, EspP, and StcE.[113, 115-118] We selected, recombinantly produced, refolded, and analyzed potentially pathogenic STEC proteins. Ten proteins have been selected using genome comparison and five proteins were eventually produced in sufficient quantities to perform more extensive complement binding experiments. None of the produced proteins had an effect on complement activation in the *in vitro* studies.

A comparative *in silico* analysis was used to select proteins encoded by genes that are only present in STEC and not in other *E. coli* genomes. For eight of the ten selected proteins, the expression and secretion was previously confirmed by other experiments.[246] Only for ECs1821 and ECs1126, no experimental confirmation for secretion was available.

Five of the selected proteins were produced recombinantly and used in complement activation and binding assays. However, none of these proteins showed effect on complement activation in human serum. In non-STEC-HUS, which is associated with mutation in genes encoding complement proteins, it is thought that complement activation occurs mostly at the endothelial surface and not just in the fluid phase.[98] This could be the case for these STEC proteins as well.

Future perspecitives

An important limitation of this study is the use of the candidate-gene approach, where genes that are not selected during the *in silico* step will never be tested in the laboratory. In the next step of this project, we are therefore going to use a different approach. We will create a STEC genome phage display library, where all STEC proteins will be expressed and tested for interaction with different complement components (Figure 10.4). Phage display technology is a high throughput and unbiased technique for identification of novel complement evasion molecules. During phage display, proteins of choice are expressed fused to capsid proteins on the surface of a filamentous Ff-phage, a virus that specifically infects the Gram-negative bacterium *E. coli* carrying F- pili. A phage library contains a large amount of different phage clones randomly displaying different proteins resulting in a heterogeneous mixture of phages. The chromosomal DNA of the pathogenic STEC strains Sakai and EDL933 will be randomly sheared and inserted into a phagemid vector lacking a native signal sequence. The presence of a signal sequence or membrane targeting motif within the fusion protein construct is essential for protein display. In a secretome phage display, the membrane targeting motif in the inserted fragment is used to specifically display secretome proteins.[247-249] The

membrane targeting motif in the inserted fragment addresses the encoded bacterial protein in fusion with a phage capsid protein PIII to the *E. coli* cytoplasmic membrane, where it is assembled into the phage particle. The displayed STEC fusion protein retains its native conformation and activity, which allows for affinity selection.

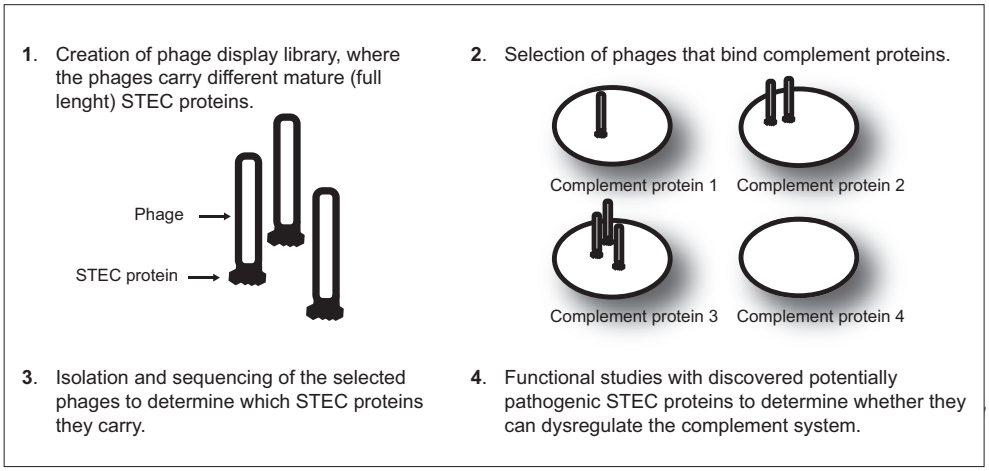

Figure 10.4. Schematic representation of the work flow in the follow-up project involving the phage display screening of mature (full length) proteins originating from the complete STEC proteome.

Phage selection will be performed by panning the phage library on different complement components: FH, FI, CD46, C3, FB, C1-C9, FD, total TCC, etc. For each complement protein, only 10 µg will be sufficient. Thus the initial screening of the phage display library for binding to complement component will be independent of the successful production of purification of proteins in high yields (mg amounts), as was the case in this study. STEC proteins that demonstrate complement binding, will be produced or purified from the STEC secretome in high quantities in order to perform more extensive complement activation tests *in vitro*. Moreover, they also will be tested *in vivo* in wild type mice and transgenic mouse models for the ability to induce HUS phenotype in the presence or absence of shiga toxins.[250, 251]

Therefore, this project will be continued using a more promising strategy for the selection of novel possible STEC virulence factors. The platform to produce and purify recombinant STEC proteins that was created during this project will be used in the follow-up study and will facilitate more efficient experimental testing.

Acknowledgements

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Chapter 11

Invasive pneumococcal disease results in renal thrombotic microangiopathy in mice: influence of virulence factors and complement factor H deficiency

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Submitted.

Abstract

Infection with *Streptococcus pneumoniae* (SP) is one of the causes of the hemolytic uremic syndrome (HUS), a rare but severe renal disorder that is thought to be caused by glomerular endothelial damage due to desialylation of glycoproteins by the pneumococcal neuraminidase. Defective complement regulation, as seen in other HUS patients, might be an important risk factor.

Wild-type mice ($FH^{+/+}$) and mice deficient in the complement regulator factor H ($FH^{-/-}$) were infected with SP. Bacterial virulence factors putatively involved in SP-HUS pathogenesis or in complement evasion and activation (pspC, pneumolysin [ply], and neuraminidase A [nanA]) were inactivated to determine if a temporally complement deficiency due to SP infection is enough to develop HUS.

Bacterial counts in blood were higher in $FH^{-/-}$ mice and these animals showed more signs of disease, higher acute kidney injury markers in urine, and a higher inflammatory response after infection. Fibrin(ogen) staining, as a marker for the presence of thrombotic microangiopathy, however, was present in similar levels in both $FH^{+/+}$ and $FH^{-/-}$ mice. The mutant bacteria Δ*pspC* and Δ*nanA* acted similar as wild-type bacteria; Δ*ply* bacteria were less virulent.

While no influence of factor H and secondary C3 deficiency was found on the level of thrombotic microangiopathy, its deficiency clearly resulted in a decreased host resistance against pneumococcal infection, as shown by higher bacterial load in blood of $FH^{-/-}$ mice. Therefore, complement deficiency might lead to higher bacteria load in patients, thereby increasing the risk for the development of HUS.

Introduction

The hemolytic uremic syndrome (HUS) is one of the main causes of acute renal failure in childhood. This thrombotic microangiopathy (TMA) manifests as hemolytic anemia, thrombocytopenia, and acute renal failure. Patients are classified according to their etiology, which can be infection induced, a disorder of the complement system, or a rare cause associated with HUS (medication, systemic diseases, pregnancy, etc.).[1, 109] In most cases in childhood, the disease is triggered by an infection with Shiga-like toxin producing *Escherichia coli* (STEC); non-STEC-HUS is seen in 5-10% of the patients.

One of the causes of non-STEC-HUS is an infection with *Streptococcus pneumoniae* (SP-HUS).[48] A higher morbidity (25-50%) and mortality (5-25%) rate has been reported over the years for this type of HUS as compared to STEC-HUS.[50, 58] In literature, these cases are associated with pneumonia and meningitis.[53] The high incidence of empyema, which is found in 51% of SP-HUS cases associated with pneumonia, suggests that a high bacterial load may increase the risk of HUS in the setting of pulmonary infection.[50] The current hypothesis of the pathogenesis of SP-HUS is desialylation of glycoproteins of red blood cells, thrombocytes, and endothelial cells by the bacteria's neuraminidase (NanA), leading to the exposure of the Thomson Friedenreich cryptantigen (T-antigen).[48, 49] Naturally occurring circulating IgM antibodies against this T-antigen, which are present in the plasma of most people, can bind to the exposed T-antigen, resulting in the agglutination of red blood cells *in vitro*. The exact role of these anti-T-antigen antibodies in the pathogenesis of SP-HUS is not known: T-antigen activation can be caused by more than 77 well-defined serotypes, but most of them are not associated with pneumococcal HUS.[51] Furthermore, no difference was seen in overall neuraminidase activity between SP-HUS isolates and control isolates.[253] This indicates that other important susceptibility factors, like a defective complement regulation seen in other non-STEC-HUS patients, have to be considered in the pathogenesis of SP-HUS.

Previously, a significant role of the alternative complement pathway has been shown in pneumococcal infection in mice deficient in C3 and factor B.[123] These mice were unable to form C3 convertases and therefore had hardly any hemolytic activity, resulting in more severe pneumococcal disease. Patients with HUS due to an alternative complement pathway disorder can still produce C3 convertases, but have an impaired complement regulation in the fluid phase and/or on the cell surface. This results in a hyperactive complement system. Increased alternative pathway activity may result in complement factor consumption and secondary deficiencies. In ~30% of these patients, a genetic aberration is found in the alternative pathway regulator FH.[29, 78] Although mice

deficient in FH still had a normal renal function after two months, within eight months they spontaneously developed dense deposit disease, another complement-mediated glomerular disease strongly associated with FH mutations.[250] A conditional trigger of the complement system might be needed to develop HUS in these mice, as is observed in patients.[71, 93]

This hypothesis has been investigated previously: Shiga toxin (Stx) was administered to mice with a heterozygous deficiency of factor H to induce STEC-HUS, but this resulted in renal tubular but not glomerular injury.[251] This is probably due to the location of the Gb3-receptor that is targeted by Stx: in mice, this receptor is only expressed in the renal tubules and not in glomeruli.[254] So, shiga toxin is not the right trigger to induce glomerular TMA in mice, but *S. pneumoniae* could be. *Streptococcus pneumoniae* does not bind mouse factor H, thus studies in $FH^{-/-}$ mice allow to study the effect of FH on overall complement activity, not influenced by FH complement interactions on the pneumococcal surface.

In earlier studies, it has been shown that pneumococci have the capacity to bind one of the main complement regulators in humans, complement factor H (FH), by the pneumococcal surface protein C (PspC), thereby improving survival in the host.[125-127] In addition, during autolysis of the bacteria, pneumolysin is released, an important virulence factor that activates the complement system, leading to complement depletion and reduced opsonisation.[131] Infection with *S. pneumoniae* might therefore lead to temporally complement deficiency, by means of evasion or activation, and loss of complement regulation. This in turn could result in less protection of the host cells to complement activation and, together with the bacteremia in the patient, in vascular injury and the symptoms of HUS.

To test the hypothesis that a defective host complement regulation is involved in the pathogenesis of SP-HUS, wild-type mice ($FH^{+/+}$) and mice deficient in complement factor H ($FH^{-/-}$) were infected with *Streptococcus pneumoniae* bacteria. The role of complement evasion and/or activation by the bacteria itself is further studied by infection with mutant bacteria lacking virulence factors associated with SP-HUS or complement [PspC (Δ*pspC*), neuraminidase A (Δ*nanA*), or pneumolysin (Δ*ply*)].

Materials and methods

Bacterial strains and growth conditions

S. pneumoniae TIGR4 (serotype 4)[255] and its respective mutants were routinely grown in Todd-Hewitt broth supplemented with 5 g/l yeast extract (THY) or on Colombia blood agar plates (Oxoid)

at 37ºC and 5% CO_2. Prior to infection, the TIGR4 wild-type and mutant strains were passaged in mice to maintain virulence as described previously.[256] Cultures of mouse-passaged *S. pneumoniae* TIGR4 strains were grown to an optical density at 620 nm of 0.2, were aliquoted, and stored at -80ºC in 15% glycerol.

Construction of pneumococcal mutants

Directed deletion mutants of *S. pneumoniae* TIGR4 lacking pneumococcal surface protein C (Δ*pspC*), pneumolysin (Δ*ply*) and neuraminidase A (Δ*nanA*) were constructed by allelic replacement of the target gene with an antibiotic resistance marker as described previously.[257] Briefly, overlap extension PCR was used to insert the spectinomycin resistance cassette of the pR412 plasmid between the two 500-bp flanking sequences adjacent of the target gene. The resulting PCR products were introduced by competent stimulating peptide (CSP-2)-induced transformation into TIGR4. Directed mutants were obtained by selective plating and were checked for correct integration of the antibiotic resistance cassette into the target gene by PCR using control primers located inside the gene. Subsequently, the TIGR4 WT strain was transformed with chromosomal DNA isolated from the mutants to prevent the accumulation of inadvertent mutations elsewhere on the chromosome. In addition, inactivation of the target gene was confirmed by qPCR. The primers (Biolegio, Nijmegen, Netherlands) used in this study are listed in Table 1.

Animals

$FH^{+/+}$ and $FH^{-/-}$ mice with a C57BL/6 genetic background were generated as described before.[250] Genomic DNA was extracted from mouse tissue (tail or ear snips) and analyzed for the presence of complement factor H by means of PCR. Three different primers were used: one is located in exon 2 (*mFH/2+*; 5'-GTAAAGGTCCTCCTCCAAGAG-3') present in all mice; the others are situated in exon 3 of the normal gene (*mFH/3-*; 5'- GGTATAAACAACCTTTGCACC-3') or in a sequence specific for the mutant gene (*neo/3-*; 5'- GGGGATCGGCAATAAAAAGAC-3'). This resulted in a WT band of 600 bp and a recombinant band of 400 bp. Further details have been published previously.[258]

Measurement of disease score

During the experiment, mice were monitored twice a day and were scored according to their condition. Used parameters were weight, ruffled coat, hunched back, reduced mobility, body temperature, and a moribund state (Table 2). Mice were sacrificed when reaching humane endpoints: i.e. an overall score of more than 15 and/or a moribund state and/or weight loss >20%

from starting weight and/or a body temperature below 35ºC was used as an indication for euthanasia.

Mouse bacteremia model

All animal procedures were performed in accordance with local and national ethical approval and guidelines. Bacteremia experiments were conducted in six-to-eight week old male mice. Mice were infected intravenously in the tail vein with $1x10^7$ colony forming units (CFU) in 100 µl sterile phosphate-buffered saline (PBS). Before infection, blood was collected via a cheek puncture and also urine secreted by the animals during fixation was collected. Mice were sacrificed 19 hours after infection, when several mice reached humane endpoints as described above.

Table 11.1. Primers used in the construction of directed deletion mutants of *S. pneumoniae* TIGR4 lacking pneumococcal surface protein C (Δ*pspC*), pneumolysin (Δ*ply*) and neuraminidase A (Δ*nanA*).

Primers for mutant generation:		
spn1923_L1	Spn -1923 ply	5'-GAAGACTTGGTCAATCCTGTC-3'
spn1923_L2	Spn -1923 ply	5'-CCACTAGTTCTAGAGCGGCCCTGATGGGTCAAGAGTTTC-3'
spn1923_R1	Spn -1923 ply	5'-AAGCTAGCCTCAGGTTGCTC-3'
spn1923_R2	Spn -1923 ply	5'-GCGTCAATTCGAGGGGTATCACCGATTTGCCACTAGTGCG-3'
spn1923_C	Spn -1923 ply	5'-CGAGAAGTGCTCCAGGATAG-3'
spn1693_L1_nanA	Spn -1693 nanA	5'-GTTCGATAAGGATTGAGCAGG-3'
spn1693_L2_nanA	Spn -1693 nanA	5'-CCACTAGTTCTAGAGCGGCCGTTCCAAATACCACTGCTC-3'
spn1693_R1_nanA	Spn -1693 nanA	5'-GGATCGAAAGTCTCATGAATAC-3'
spd1504_R2	Spn - 1693 nanA	5'-GCGTCAATTCGAGGGGTATC-GCTTCACTAGGACTAACAGC-3'
spd1504_C	Spn - 1693 nanA	5'-TGGCATCTGGCTTAAACTCC-3'
spn2190_L1	Spn -2190 pspC	5'-TTG AGG CAA TGG TGC ACA AG-3'
EMspn2190_L2	Spn -2190 pspC	5'-CCACTAGTTCTAGAGCGGCTACACTAGCTACTCCAACAC-3'
EMspn2190_R1	Spn -2190 pspC	5'-CAAGATGAAGATCGCCTACG-3'
EMspn2190_R2a	Spn -2190 pspC	5'-GCGTCAATTCGAGGGGTATCGCTATGGAGTCAATGCCAAT-3'
EMspn2190_C	Spn -2190 pspC	5'-TCGTTCTCTGTCGCATGAAC-3'
Primers for qPCR:		
2190pspC_Fw_qpcr	Spn 2190 pspC	5'-CATCTTCTTCGTCAAGTTCAGACTCT-3'
spn_2190pspC_Rev_qpcr	Spn 2190 pspC	5'-CTGGTTCTGTCGGCTTGTTTG-3'
HB_Sp_plyF	Spn -1923 ply	5'-TCGTAGTGGATGAGACCTTGTTAGA-3'
HB_Sp_plyR	Spn -1923 ply	5'-GCCAAACCAGGCAAATCAAT-3'
HB_Sp_nanAF	Spn - 1693 nanA	5'-TTGGAACGTCTCCTGTTTTAGCT-3'
HB_Sp_nanAR	Spn - 1693 nanA	5'-CCCCGAAAGTTGAGTTTCATTT-3'
HBgyrAF	Spn – 1219 GyrA	5'-AATGAACGGGAACCCTTGGT-3'
HBgyrAR	Spn – 1219 GyrA	5'-CCATCCCAACCGCGATAC-3'
PBMrTn9	$Spec^{res}$ cassette	5'-CAATGGTTCAGATACGACGAC-3'
PBpR412_L	$Spec^{res}$ cassette	5'-GCCGCTCTAGAACTAGTGG-3'
PBpR412_R	$Spec^{res}$ cassette	5'-GATACCCCTCGAATTGACGC-3'

Abbreviations: Spec, spectinomycin resistance cassette; L1 and L2, primers downstream of the open reading frame of the target gene; R1 and R2, primers upstream of the open reading frame of the target gene; C, control primer within the target gene. Ply, pneumolysin; nanA; Neuraminidase A; pspC, pneumococcal surface protein C.

Bacteria were cultured from blood obtained by retro-orbital puncture. One kidney was used for histology; half of it was fixed in 10% buffered formalin and embedded in paraffin for light microscopy, the other half was snap-frozen for immunofluorescence studies. Urine was collected from the bladder of the animals. For bacterial quantification, obtained blood was serially diluted by 10-fold in sterile PBS and plated onto blood agar plates. Following overnight incubation at 37ºC and 5% CO_2, CFU were counted.

Measurement of renal injury markers

Kidney injury marker 1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) were measured as markers for acute kidney injury in pre- and post-infection urine samples. Commercially available ELISA assays were used (R&D Systems). As one of the signs of invasive pneumococcal disease in mice is the loss of urine production due to reduced fluid intake, it was not possible to obtain post-infection urine from all animals. Therefore, urine samples were pooled per category. No urine was available at all for $FH^{+/+}$ mice infected with WT bacteria.

Measurement of IL-6 and MIP-2 as inflammation marker

To investigate the immune response in mice infected with *S. pneumoniae* TIGR4, the general inflammatory markers interleukin 6 (IL-6) and macrophage inflammatory protein 2 (MIP-2) were

Table 11.2. Legend for disease scores. An overall score of more than 15 and/or a moribund state and/or weight loss >20% from starting weight and/or a body temperature below 35.5ºC is an indication for euthanasia.

Condition	Score	Description
Normal	0	No signs of disease
	1	Showing signs of a ruffled coat
Ruffled coat	2	Dull ruffled coat, mildly seen around neck and back
	3	Ruffled coat
Hunched back	2	Hunched back, mildly seen
	3	Hunched back
Temp. <35.5ºC	5	
Reduced mobility	3	Less mobile but still being active and reacts to any handling
	4	Back legs are walking weird and slower
	5	Not walking around much, needs to be pushed to get going
Weight loss	2	>5% body weight loss from T=0
	3	>10% body weight loss from T=0
	5	>15% body weight loss from T=0
	15	>20% body weight loss from T=0
Moribund state	15	

measured in post-infection serum samples using commercially available ELISA assays (Mouse IL-6 ELISA, eBioscience; Mouse CXCL2/MIP-2 Quantikine ELISA Kit, R&D Systems).

Histological studies

Immunostainings were performed in a standard manner.[259] To identify glomerular inflammation, the following primary antibodies were used: anti-mouse Ly-6G and Ly-6C (Pharmingen, Erembodegem, Belgium), F4/80 (Serotec, Oxford, UK), and anti-CD3 (Thermo Fisher Scientific, Rockford, IL, USA). The presence of apoptosis was investigated with an anti-active caspase 3 antibody (Cell Signaling Technology, Beverly, MA, USA). Glomerular complement deposition was demonstrated with an anti-C3c antibody (Dako, Glostrup, Denmark). The deposition of fibrin(ogen), and therefore the existence of glomerular thrombotic microangiopathy, was examined with an anti-mouse fibrin(ogen) antibody (Accurate Chemical & Scientific, Westbury, NY, USA) as described before.[259]

Fibrin(ogen) and C3 deposition were scored in 50 glomeruli by a pathologist without knowledge of the genotype of mice and treatment on a scale of 0 to 3 (0, absent; 0.5, mild; 1, moderate; 2, severe; 3, very severe). The total deposition score per mouse was expressed as the mean of the individual glomerular scores.

Statistical analysis

We used a Mann-Whitney test, with a Bonferroni correction to correct for multiple testing, to analyze bacterial counts, signs of disease, and IL-6 levels. An unpaired t-test was used to analyze KIM-1 and NGAL levels in urine samples.

Results

Mice deficient in factor H show more signs of disease and have higher bacterial counts in blood

To assess the influence of the most important complement regulator, factor H, on the host defense against pneumococcal infection, $FH^{+/+}$ and $FH^{-/-}$ mice were infected with *S. pneumoniae* TIGR4 wild-type bacteria or its mutants (Δ*pspC*, Δ*nanA*, and Δ*ply*). Wild-type animals showed less signs of disease 19 hours after infection (ruffled coat, hunched back, reduced mobility, and/or weight loss; Figure 11.1A) and had lower bacterial counts (3- tot 280-fold less) in blood (Figure 11.1B) than $FH^{-/-}$ animals, irrespective of the pneumococcal variant used bacteria

The influence of the virulence factors PspC, neuraminidase and pneumolysin on infection of $FH^{+/+}$ and $FH^{-/-}$ mice was investigated in the same experiment. In both $FH^{+/+}$ and $FH^{-/-}$ mice, Δ*ply* bacteria

were less able to survive in the bloodstream, as shown by lower bacterial counts in blood (Δ*ply* vs WT: *P*<0.0125).

C57Bl/6 mice show acute kidney injury after invasive infection with S. pneumoniae

KIM-1 and NGAL are early indicators of acute kidney injury (AKI), with an earlier detection of AKI than for instance serum creatinine.[260] All mice, irrespective of the factor H level, showed increased NGAL and KIM-1 levels after infection with wild-type or mutant *S. pneumoniae*, except for KIM-1 levels in the $FH^{+/+}$ mice infected with Δ*ply* (Figure 11.2). After infection with wild-type or mutant TIGR4, NGAL is significantly more increased in $FH^{-/-}$ mice, but no clear trend is seen in KIM-1 levels. Both KIM-1 and NGAL are significantly lower in $FH^{+/+}$ and $FH^{-/-}$ mice after infection with Δ*ply* compared to the other bacteria (*P*<0.01).

Higher levels of inflammatory cytokines in FH deficient mice after invasive S. pneumoniae *infection*

To investigate the role of FH deficiency in the immunological response after *S. pneumoniae* infection, the cytokines IL-6 and MIP-2 were measured (Figure 11.3). In mice infected with WT TIGR4 and Δ*ply*, both IL-6 and MIP-2 levels were higher in $FH^{-/-}$ mice than in $FH^{+/+}$ mice 19 hours after infection; in Δ*nanA* infected mice, this was only the case for IL-6. Both inflammatory cytokines were

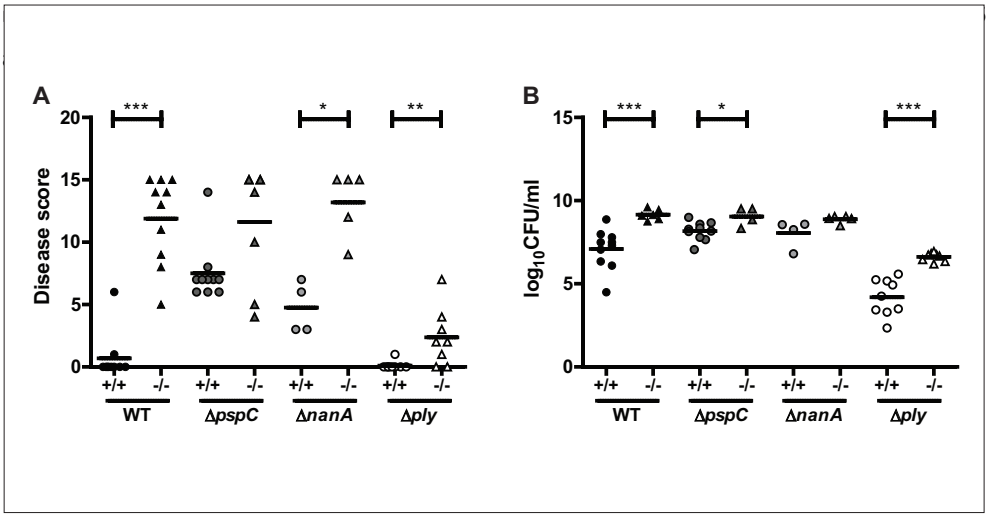

Figure 11.1. Disease scores **(A)** and bacterial load in the blood **(B)** of mice 19 hours after intravenous infection with $1x10^7$ CFU of wild-type *S. pneumoniae* or its respective Δ*pspC*, Δ*nanA*, or Δ*ply* mutant. Each point depicted indicates one mouse; the horizontal line represents the mean. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

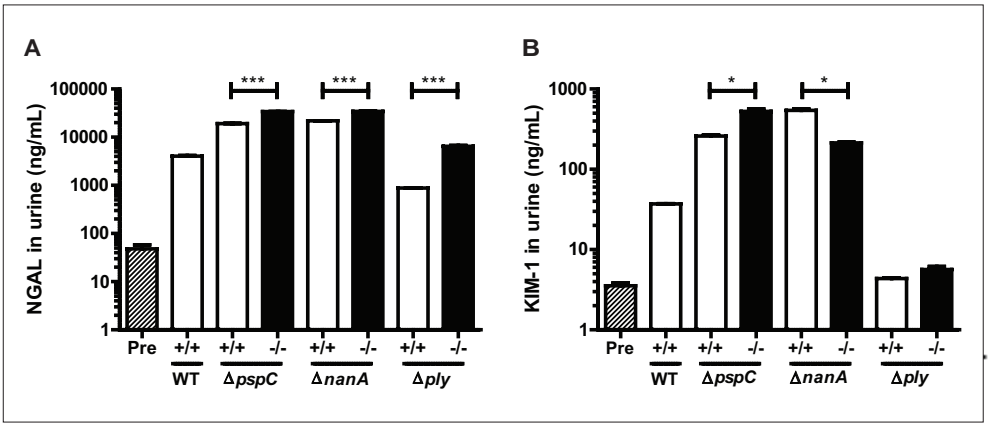

Figure 11.2. Levels of acute kidney injury markers NGAL **(A)** and KIM-1 **(B)** in pooled pre-infectious (striped bars) and pooled post-infection urine samples in $FH^{+/+}$ (grey bars) and $FH^{-/-}$ mice (black bars) mice infected wild-type *S. pneumoniae* or its respective Δ*pspC*, Δ*nanA*, or Δ*ply* mutant. Post-infection urine samples were collected 19 hours after infection. No urine samples were available from $FH^{-/-}$ mice infected with wild-type bacteria. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

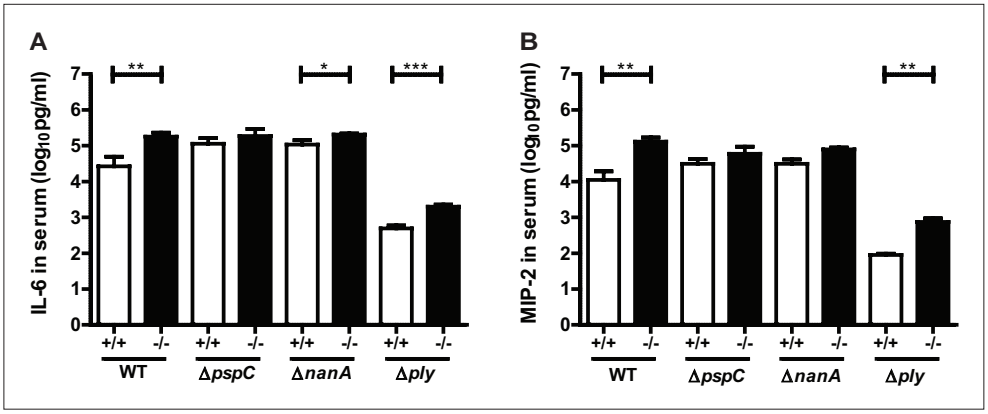

Figure 11.3. Post-infection levels of inflammatory cytokines IL-6 **(A)** and MIP-2 **(B)** in serum samples of $FH^{+/+}$ (open bars) and $FH^{-/-}$ (black bars) mice infected wild-type *S. pneumoniae* or its respective Δ*pspC*, Δ*nanA*, or Δ*ply* mutant. Post-infection serum samples were collected 19 hours after infection. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

C57Bl/6 mice develop thrombotic microangiopathy after invasive streptococcal infection

The influx of granulocytes, T-cells, or macrophages into the glomerulus and the presence of apoptosis was not influenced by FH status or virulence factor (data not shown). Initial complement C3 levels and C3 deposition in glomeruli of $FH^{-/-}$ mice and not in $FH^{+/+}$ mice was comparable with that observed in previous experiments (data not shown).[250] This deposition was not influenced by the depletion of the different TIGR4 virulence factors (data not shown). To evaluate the presence of glomerular thrombotic microangiopathy, renal tissue was examined for the presence of fibrin(ogen), the endpoint of the coagulation cascade after endothelial damage. Antifibrin(ogen) immunostaining

showed moderate fibrin(ogen) deposition (score >1) in the glomeruli of 7 $FH^{+/+}$ mice and 6 $FH^{-/-}$ mice infected with different bacteria (Figure 11.4). Although $FH^{-/-}$ mice infected with Δ*pspC* had significantly less fibrin(ogen) staining than $FH^{+/+}$ mice, this was not statistically significantly different compared to mice infected with wild-type bacteria.

Discussion

To investigate the role of the complement system in the pathogenesis of SP-HUS, we induced pneumococcal infection in wild-type mice ($FH^{+/+}$) and in mice deficient in the complement regulator factor H ($FH^{-/-}$). Several virulence factors of *S. pneumoniae* involved in SP-HUS pathogenesis or in complement evasion and activation (PspC, pneumolysin, and neuraminidase) were inactivated, to determine if a temporally complement deficiency due to *S. pneumoniae* infection is leading to the development HUS.
Mice deficient in factor H with secondary C3 deficiency were less capable in clearing bacteria, showed more acute kidney injury, and had a stronger immunological response after infection than wild-type mice. The observed thrombotic microangiopathy in 23% of the mice, however, was not associated with complement deficiency. Infection with the knock out mutants Δ*pspC* and Δ*nanA* resulted in similar disease phenotype as wild-type pneumococci, while Δ*ply* was less virulent in both $FH^{+/+}$ and $FH^{-/-}$ mice.

After infection of $FH^{+/+}$ and $FH^{-/-}$ mice with *S. pneumoniae*, it was shown that a defective host complement regulation influences the host response against pneumococcal infection. Bacterial counts were higher in $FH^{-/-}$ mice and these animals showed more signs of disease (Figure 11.1). Previously, C57Bl/6 mice deficient in factor B had increased susceptibility *S. pneumoniae* as well.[261] FH levels had no influence on phagocyte and/or lymfocyte influx in the glomerulus of the mice, but the acute kidney injury marker NGAL was higher after infection in $FH^{-/-}$ mice; no trend was visible for KIM-1 levels. This might be due to the time it was measured: in humans with postoperative AKI, the concentration of NGAL is highest within 2 hours after surgery, while KIM-1 has only significantly increased after 18 hours.[260, 262]

The presence of fibrin(ogen) was seen in 23% (13/57) of the mice after infection with wild-type or mutant bacteria, but this was not associated with the presence of absence of FH: both $FH^{+/+}$ and $FH^{-/-}$ mice infected with the different pneumococcal variants were diagnosed with renal TMA (Figure

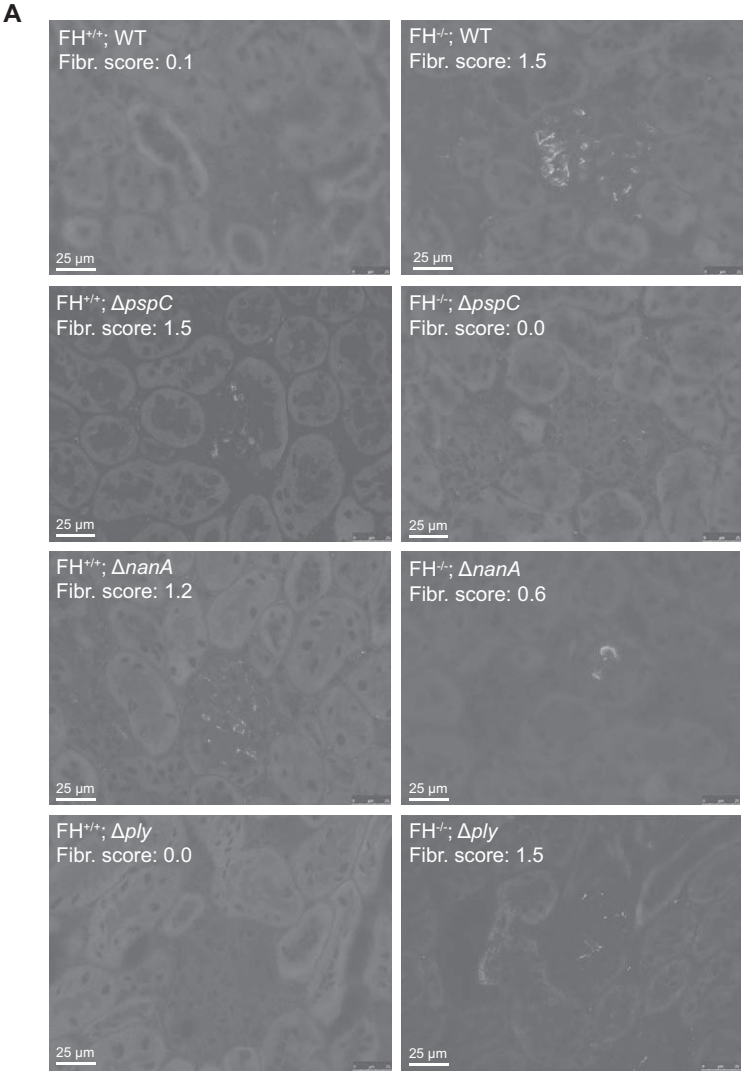

Figure 11.4. Thrombotic microangiopathy is present in the glomeruli of mice infected with *S. pneumoniae*. Renal sections were stained **(A)** and semiquantitatively scored **(B)** for the presence of fibrinogen. In figure 11.4B, each point depicted indicates one mouse; the horizontal line represents the mean. *, $P < 0.05$. AU, arbitrary units.

11.4). Mice deficient in factor H that were not infected with pneumococci did not show TMA, indicating that this is likely a result of the infection.[250] Renal histological studies in mice models of pneumococcal infection are scarce. In one study, both wild-type C57Bl/6 mice and mice deficient in selectin, involved in leukocyte rolling along the endothelium but with a normal complement activation, did show thrombosis in spleen and liver, but not in kidney.[263] In another study examining the effect of complement deficiency on pneumococcal infection associated coagulation, the spleen of wild-type and $C1q^{-/-}$ mice did show multiple (micro)thrombi after infection with serotype 6A, while in $C2/FB^{-/-}$ mice, with no complement activity at all, no thrombi were formed; unfortunately, this study did not include renal histology.[264] These results indicate that the alternative complement pathway contributes to the formation of splenic TMA after pneumococcal infection. Our factor H deficient mice did not show alternative pathway activity in a hemolysis assay (data not shown), like the $C2/FB^{-/-}$ mice, which is likely due to a hyperactive complement system. It is known that the C3 convertase C3bBbP and the membrane attack complex (sC5b-9) are involved in the cleavage of pro-thrombin into active thrombin, which is needed for the formation of a stable fibrin clot.[265, 266] These convertases will not be present in serum of FB deficient mice, in contrast to serum of $FH^{-/-}$ mice. Additionally, atypical HUS patients with FH mutations, resulting in a dysregulated alternative complement pathway, have increased levels of C3BbP and sC5b-9 in the acute phase of disease.[237]

Based on the results of the infection experiments with the mutant bacteria, we cannot elucidate on the role of complement evasion and/or activation by *S. pneumoniae*. Pneumolysin seems to be a more important general virulence factor than neuraminidase or PspC, but as the decreased survival of Δ*ply* bacteria was alike in $FH^{+/+}$ and $FH^{-/-}$ mice, the pathogenic mechanism is probably not via the binding of the alternative pathway regulator.

The current hypothesis of SP-HUS pathogenesis involves T-activation due to desialysation of glycoproteins by pneumococcal neuraminidase, leading to *in vitro* hemagglutination, but we did not observe decreased TMA formation in mice infected with Δ*nanA* pneumococci. The role of T-activation in pneumococcal infection in mice is not fully understood, but neuraminidase can expose cryptic T-antigens on murine lymphocytes.[267] It is known that TIGR4 can produce at least one other neuraminidase (NanB), which in other serotypes can compensate for the absence of NanA.[268] A third neuraminidase, NanC, has been associated with SP-HUS as well[269], but this gene has not been identified in *S. pneumoniae* TIGR4 yet. It would be interesting to perform the same experiments with *S. pneumoniae* without any of the neuraminidases.

In contrast to a previous study, we did not observe a significant reduction of Δ*pspC* bacteria in the bloodstream of C57Bl/6 mice.[270] However, in this study mice were infected with lower numbers of bacteria ($1x10^4$ CFU), while we used a significantly higher infectious dose ($1x10^7$ CFU), which makes it difficult to compare the results. BALB/c mice infected with comparable, higher, amounts of bacteria of pneumococcal strain D39 lacking PspC displayed similar bacterial levels as wild-type D39 as well.[271, 272] PspC is considered to be the most important human factor H binding protein of *Streptococcus pneumoniae*, but there are contradictory opinions about the binding capacity to mouse factor H.[270, 271] It is known that human factor H interacts via two contact sites with PspC located in SCR8-11 and SCR19-20.[127, 273] These SCRs are not homologous to mouse factor H: four out of six residues in SCR20 involved in human FH binding by *S. pneumoniae* are not identical (human: R1182, R1203, R1206, R1210; mouse: K1182, Y1203, Y1206, D1210).[273] Indeed, none of the 11 different capsular serotypes tested in the past showed detectable binding to mouse FH, although TIGR4 was not examined.[271] To study the interactions of PspC with human FH *in vivo*, and thereby its role in host response and in the pathogenesis of SP-HUS, a transgenic mouse model is needed, with the human binding sites for PspC and murine complement regulatory and surface-binding domains to preserve complement regulation.

The alternative complement pathway indeed seems to be important in the clearance of pneumococci, as shown by increased bacterial blood counts in $FH^{-/-}$ mice. However, this higher bacterial blood count was not associated with TMA in mice. The factor H deficiency in $FH^{-/-}$ mice might have resulted in reduced complement-mediated tissue inflammation hence kidney injury similar to wild-type mice despite higher bacterial counts. This might be different in humans, as patients with pneumonia and empyema, and therefore a higher bacterial load, have a higher risk to develop HUS.[50] An impaired host response due to a factor H deficiency, as shown in our experiments, might therefore result in increased SP-HUS risk. Indeed, we and others[91] identified genetic and/or acquired complement abnormalities in SP-HUS patients that are associated with a dysregulated complement system, thereby possibly contributing to the development of the disease.

In summary, invasive *Streptococcus pneumoniae* infection alone is capable to induce glomerular thrombotic microangiopathy. This was not associated with a dysregulated complement system due to a factor H deficiency: both $FH^{+/+}$ and $FH^{-/-}$ mice were diagnosed with glomerular TMA. The tested virulence factors pneumolysin, neuraminidase A, and PspC had no clear effect on the thrombotic microangiopathy. The role of other virulence factors, including other neuraminidases than NanA,

warrants further investigation. Factor H deficiency does result in an impaired host defence response, as shown in our experiments by higher bacteria load in blood of in $FH^{-/-}$ mice. Complement deficiency might lead to higher pneumococcal load in patients as well, thereby increasing the risk for SP-HUS.

Acknowledgements

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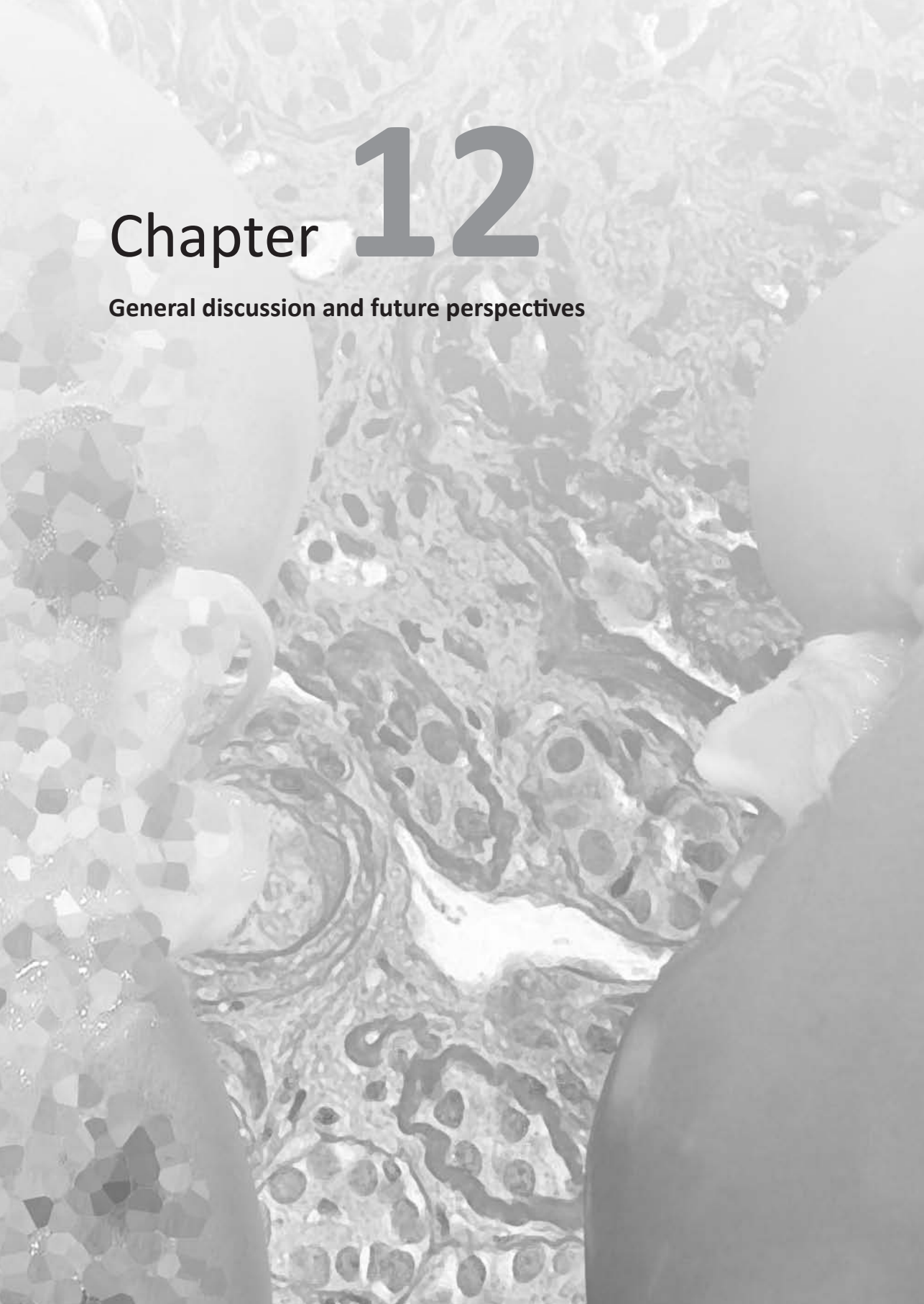
Closing remarks



Chapter 12

General discussion and future perspectives

Content

Since the first use of the term hemolytic uremic syndrome (HUS) for children with renal failure, hemolytic anemia, and thrombocytopenia, the pathogenesis of the disease has been studied extensively. In the mid eighties of the twentieth century, an infection with shiga-like toxin producing *Escherichia coli* (STEC) was identified as the main cause of diarrhea-associated HUS. Twenty years later, the role of the complement system as etiology in atypical HUS (aHUS) was clearly demonstrated. In childhood, STEC-HUS and complement-mediated HUS, the latter here called aHUS, are the main causes of HUS, together with HUS induced by an *Streptococcus pneumoniae* infection (SP-HUS).

Since the discovery of mutations in complement factor H in aHUS patients in 1998, five other complement genes have been associated with the disease, which all resulted in an impaired regulation of the alternative complement pathway. Mutation in three genes ecoding proteins of the coagulation system have been found in aHUS patients as well. Nowadays, in more than 50% of the aHUS patients mutations in complement genes or the presence of autoantibodies against factor H (αFH) have been identified. The role of the complement system in infection-induced HUS has not been fully elucidated yet, but microbiology studies have shown that both bacteria that are associated with the disease, STEC and *Streptococcus pneumoniae*, can protect themselves against the complement system by binding complement regulators and their toxins are able to activate the complement system.

The purpose of this thesis was to further elucidate the role of the complement system, in particular the alternative pathway, in the pathogenesis of STEC-HUS, SP-HUS, and aHUS. We showed that, compared to age-matched controls, the complement system is indeed activated in the acute phase of disease in all HUS subgroups: the alternative pathway activation markers C3bBbP, C3b/c, and the C3d/C3 ratio were increased in the acute phase of disease and normalized in remission. The activation products were more increased in aHUS patients than in infection-induced HUS patients. Therefore, these markers probably may not only be used for monitoring disease activity, but can be used to distinguish between different HUS etiologies at time of presentation as well. Mutations in aHUS associated genes and/or αFH were present in STEC-HUS and SP-HUS patients as well, albeit in a lesser amount. The influence of these complement aberrations on the disease course and outcome in STEC-HUS and SP-HUS needs to be investigated in larger cohorts.

12.1. The complement system and aHUS

With the identification of mutations in complement (regulating) genes in familial and sporadic aHUS patients, the pathogenic mechanisms of disease can nowadays be explained in more than 50% of these aHUS patients. Our aHUS cohort with 70 patients was screened for potentially pathogenic genetic aberrations in the associated genes *CFH*, *CFI*, *CD46*, *CFB*, *C3*, *CFHR5*, *THBD*, and *DGKE*, and for the presence of αFH (see Chapter 2, Chapter 3, Chapter 4, and Chapter 6 of this thesis).[72, 77, 93, 94] In 52,8% (37/70) of the patients a potential disease causing nonsynonymous rare variant or αFH have been identified. For the first time to our knowledge, we showed the presence of mutations in more than one associated gene in aHUS patients (*CFI* and *CD46*: 2 patients; *CFI* and αFH: one patient)[72], a finding that has been confirmed by others.[76, 78] In the remaining patients, aHUS could not be explained by a mutation in any of the screened genes.

The association of aHUS with new genes encoding proteins that are not solely involved in the complement system, but also in the coagulation system, implicate that not only the complement system is an important contributor in the pathogenesis of aHUS. In DGKE-associated HUS, the thrombotic microangiopathy even seems to result from impaired proliferation and repair of endothelial cells and impaired angiogenesis rather than from complement-mediated cell damage.[274] So far, mutations have been identified in three coagulation genes (*DGKE*, *THBD,* and *PLG*), but isolated mutations in the last two genes are rare: for instance, in a French cohort of 214 patients, none had a mutation in only *THBD*, and in *PLG,* three out of four patients with a mutation had a deleterious nonsynonymous rare variant in a complement gene as well.[76, 85, 275] All described patients with mutations in *DGKE* were diagnosed with aHUS within the first year of their live and in all but one patient hypertension, microhematuria, and proteinuria (even nephrotic syndrome) persisted after renal function recovery, hereby distinguishing this specific subgroup of patients from patients with complement mutations.[83, 84] Interestingly, all *DGKE* patients originated from countries around the Mediterranean Sea (Spain, Northern Africa, Turkey, Italy), possibly suggesting a founder effect. In our aHUS cohort, including eight patients with an early-infantile onset of disease, we could not find any potentially pathogenic sequence variations in *DGKE* and only one in *THBD*. We did not screen *PLG* in all our patients yet; anyhow, the coverage on a HiSeq2000 sequencer commonly used in whole exome sequencing (see Chapter 5) was too low to exclude the presence of mutations in *PLG* in that patient group (a horizontal coverage of 68.4%; 13 out of 19 coding exons vertically covered with >10 reads throughout the exon).

So far, mutations have been found in aHUS patients in almost ten genes encoding proteins of both the complement system and the coagulation pathway. Genetic screening of all associated genes and haplotypes via regular methods (PCR and Sanger sequencing) is labor intensive, costly, and time consuming. Next generation sequencing has already resulted in two new genes that could be associated with the disease (*DGKE*[83, 84] and *PLG*[85]), but in our experience, the sole application of whole exome sequencing does not result in a higher diagnostic yield than conventional sequencing, as shown in Chapter 5, as exon 23 of *CFH* and most coding regions of the *CFHR* genes are not fully covered. At this moment, next generation sequencing in combination with conventional amplicon based (Sanger) sequencing of the missing *CFH* region and the deep intronic SNPs of aHUS associated haplotypes and multi-ligand probe amplification for detection of *CFH/CFHR* rearrangements and deletions can be used to maintain or even improve current diagnostic yields in a more efficient manner.

The incomplete penetrance rate of ~60% seen in aHUS patients indicates that additional triggers, genetic and/or environmental, are probably needed for the disease to developed.[29] The presence of associated risk haplotypes in *CFH*[79] and/or *CD46*[80] have been associated with the penetrance of disease in HUS families. Goicoechea de Jorge *et al*. (2007) showed that all HUS patients in a family with a *CFB* mutation (p.Phe286Leu) also presented the $CD46_{ggaac}$ haplotype, while in the healthy carriers only one individual, who is probably still at risk for development of HUS, harbored the *CD46* haplotype.[74] In three families with a *CFH* mutation (p.Arg1215Gly), Sansbury *et al*. found that the *CFH* haplotype on the allele not carrying the *CFH* mutation had a significant effect on disease penetrance.[92] These results and the presence of combined mutations indicate that a thorough mutational screening of all associated genes, including the associated risk SNP haplotypes, is needed to be able to give family members a proper genetic advice about the chances to get the disease as well. This is nicely shown in an aHUS patient that was diagnosed with three recurrence within 10 years, including in two renal grafts.[157] Genetic screening of the genes encoding FH, FI, and CD46, the aHUS associated genes at time of investigation, revealed a familial heterozygous pathogenic mutation in *CFI* (c.1019T>C; p.Ile340Thr)[158], as depicted in Figure 12.1. None of the other family member ever showed any aHUS symptoms. After the discovery of new aHUS genes and haplotypes, the genetic screening of all family members was extended. Next to the *CFI* mutation, the aHUS predisposing missense variation p.Arg161Trp in *C3* [94, 175] was identified in both the father and the index patient. The *CFH* haplotype[71] was present in all family members, but not in the mother; the

CD46 haplotype[80] was seen in the mother, the two elder sisters, and the index patient (Figure 12.1). A cousin of the father (daughter of his father's sister) died of HUS at the age of 25, but no DNA material was available to investigate. Interestingly, only the index patient carried all four genetic aberrations. This indicates that the presence of all four genetic changes, including the aHUS associated haplotypes, seems to be needed within this family to develop aHUS. This risk probably will be smaller in family members with for instance two out of four genetic aberrations, although it can still be that a trigger with an extreme complement activation as a result (i.e. pregnancy, certain drugs) could induce the disease, as complement regulation is impaired due to the *CFI* mutation.

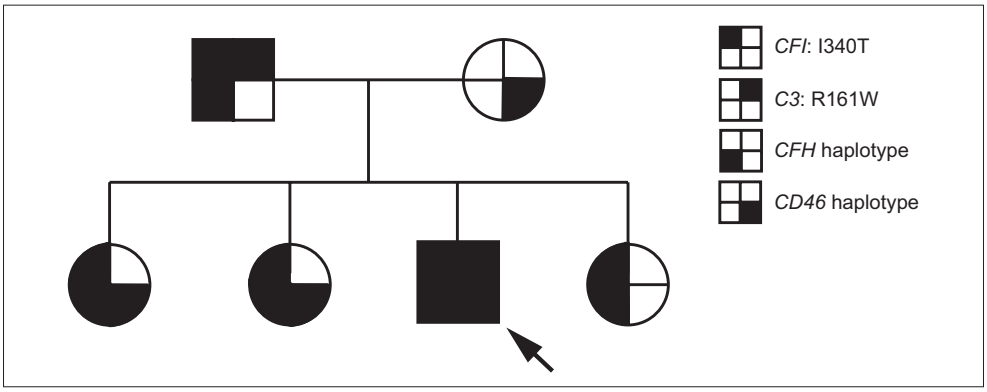


Figure 12.1. Results of genetic screening of all associated genes and SNP haplotypes[79, 80] in a family with familial aHUS. The index patient, indicated with an arrow, is the only family member that carries all four genetic aberrations (missense variations in *CFI* and *C3* and both haplotypes in *CFH* and *CD46* associated with disease).

Extra predisposing genetic factors that increase the risk for developing aHUS might not only be present in for instance complement haplotypes or complotypes, but also in changes in the endothelial glycocalyx, via which the complement regulator factor H bind to host cells. Proper binding of FH to the glycosaminoglycan heparin sulphate is required for optimal C3b regulation and it was shown that mutations in the C-terminus of FH can alter the binding properties.[212, 276] Mutations or polymorphisms in the gene encoding heparan sulphate itself (*HPSG2*) could have the same effect.[277, 278] In ten patients with familial HUS, however, no potentially pathogenic sequence variations were identified by means of WES in *HPSG2* or any other endothelial matrix component on the candidate list (see Chapter 5), but a larger cohort needs to be screened to further investigate this hypothesis.

The role of mutations in genes encoding complement (regulating) proteins in the pathogenesis of aHUS has clearly been established. Resulting complement dysregulation, leading to glomerular

endothelial cell damage, seems to be the central element. Recently it has been shown that this dysregulation mostly occurs on the level of the glomerular endothelium[98], but the presence of complement activation products in the circulation indicate that activation is also present in the fluid phase (Chapter 8).[237] Functional analysis of serological complement activation in aHUS patients, however, remains very limited. C3 and C4 levels, regularly used in clinical practice, can be normal, just like the activity of the alternative and classical complement pathway and levels of factor H and factor I (see Chapter 6 and Chapter 9). We performed a comprehensive analysis of complement activation in aHUS patients in the acute phase of disease and in remission. The alternative pathway activation markers C3d (especially the C3d/C3 ratio), C3bBbP, and C3b/c were increased in the acute phase of disease, but not in the remission phase (see Chapter 8 and Chapter 9). This normalization of alternative pathway activation existed even in patients with a clear complement dysregulation due to genetic aberrations, as shown in Chapter 8. The proposed method to monitor the deposition of C3 and C5b-9 on unstimulated human microvascular endothelial cells (HMVEC)[98] is labor intensive and technically complex. The assays detecting fluid phase complement activation we investigated, especially the C3d/C3 ratio, are more standard and easy to use and could therefore be used in early detection of a relapse of aHUS, especially in patients undergoing complement inhibition therapy.

12.2. The complement system and STEC-HUS

In the past, complement activation has been shown in patients with STEC-HUS in the acute phase of the disease.[64, 65, 110-112, 279, 280] Complement levels in these patients have been compared to adult controls in most cases, or to only a very small amount of pediatric controls that were not age-matched. The results in Chapter 9 of this thesis corroborate these anecdotal reports: we demonstrated that, compared to age-matched controls that had been collected in a strict and protocolled manner, the complement system is indeed activated in a large cohort of almost 30 STEC-HUS patients in the acute phase of disease, but not in the convalescent phase. As in aHUS patients, the C3d/C3 ratio gives the best discrepancy between the acute and convalescent phase in infection-induced HUS and could be therefore be used as a marker to monitor disease activity in HUS of any etiology. Furthermore, the investigated alternative pathway activation products C3d/C3 ratio, C3bBbP, and C3b/c were significantly more increased in aHUS patients than in STEC-HUS patients and may be used as a biomarker to distinguish at admission between these two diseases, which sometimes can be difficult solely based on clinical symptoms.

In Chapter 9, we showed that not only in aHUS patients a genetic or acquired complement aberration is present in an associated complement protein, but also in 28.0% (7/25) of the investigated STEC-HUS patients (*CFH*: n=2; *CD46*: n=1; *C3*: n=2; αFH: n=3). Some case reports on STEC-HUS patients with complement mutations have been published in the past, but these reports usually describe one or two patients with a severe disease course[87-90, 281]; no larger cohorts have been published so far. The high mutation rate seen in 27 STEC-HUS patients investigated in Chapter 9 therefore needs to be confirmed in other patient cohorts and detailed clinical follow-up data are needed to determine the influence of complement aberrations on disease course and outcome in STEC-HUS. The presence of complement aberrations might partly explain why only 5-15% of the people infected with STEC eventually develop HUS. A perfect cohort to test this hypothesis is the cohort of patients in the German outbreak: more than 4000 individuals were infected with STEC O104, but eventually less than 1000 of them developed HUS.
The outcome of disease in STEC-HUS patients with mutations or αFH in our cohort was good and the disease course was not different as in other STEC-HUS patients. However, it needs to be examined if patients carrying complement aberrations indeed have a higher risk for a more severe disease course, more sequelae, or recurrences, as they might be more susceptible anyhow to develop HUS based on their complement aberration. In several STEC-HUS patients whom had a recurrence in their native kidneys or after transplantation, which is usually not seen in STEC-HUS, complement aberrations have been identified.[87, 90] Based on our results, it is therefore advised to also screen STEC-HUS patients for complement aberrations to understand its role in pathology.

The alternative pathway activation we identified in STEC-HUS patients could be caused by complement binding or activation by the bacteria's toxins: it has been shown that Stx1 can activate C3 and Stx2 can bind the main complement regulator Factor H.[113, 116] The used Stx concentration used in these experiment, though, were much higher than the levels ever documented in STEC-HUS patients.[14] Several proteins expressed by STEC have been associated with complement activation as well, but the carefully selected ten STEC O157:H7 proteins investigated in Chapter 10 did not show this capacity. No interaction between complement proteins and produced STEC proteins was observed and none of the recombinant proteins could show complement activation in normal human serum. As the proteins that were not identified in the *in silico* selection procedure could still influence the complement system, a more robust approach is needed to analyze complement dysregulating properties of the STEC secretome. Expressing all mature proteins in the complete STEC genome using a STEC phage display library and test them for interaction with complement

components is efficient way to analyze the influence of STEC on complement activation in both the fluid phase and on the endothelial cell surface. The platform to produce and purify recombinant STEC proteins that was created in Chapter 10 can be used in this STEC phage display library study.

As the direct and indirect action of Stx on glomerular endothelial cells remains the most important factor in the pathogenesis of STEC-HUS at this moment, Stx is being targeted in the development of new treatment options. The most promising drug seems to be Shigamabs™ (Thallion Pharmaceuticals/BELLUS Health Inc., Quebec, Canada), which consists of two monoclonal antibody against Stx1 and the A subunit of Stx2, respectively, thereby blocking the inhibition of protein synthesis in the ribosome *in vitro* and altering the overall cellular distribution of Stx2.[282] The first phase II trial with 22 patients with STEC infection showed a good safety and tolerability profile and a potential benefit in preventing complications, including the development of STEC-HUS: only one patient (4.5%) progressed to HUS.[283] A phase III efficacy trial is being planned.

12.3. The complement system and SP-HUS

The pathogenesis of SP-HUS is not as extensively investigated as that of STEC-HUS and aHUS. The current hypothesis involves T-antigen exposure on erythrocytes, platelets, and glomerular endothelial cells by the pneumococcal neuraminidase, in particular neuraminidase A (NanA).[284] However, neuraminidase activity is also seen in patients with pneumococcus-associated anemia and uncomplicated invasive pneumococcal disease.[285] In one SP-HUS patient, no anti-T-antigen antibodies could be shown throughout the disease at all.[286] As the exact role of T-activation by neuraminidase *in vivo* is not clear and as we could not link NanA to the development of TMA in mice with and without FH, as shown in Chapter 11, this implicates that T-activation is most probably required but not sufficient to induce TMA and SP-HUS: other environmental and/or genetic factors in host or micro-organism have to be present as well, for instance an altered complement activity as seen in aHUS patients. In the set-up of our mice experiments (Chapter 11), we could not show a relation between the pneumococcal pneumolysin or PspC expression, associated with complement activation[131] or dysregulation[127], and the formation of thrombotic microangiopathy (TMA) in murine glomeruli.

Next to T-activation, other mechanisms for neuraminidase in the pathogenesis of SP-HUS have been proposed as well. Polyanions on the cell membrane of host cells, including sialic acids, are crucial for

the regulation of the complement system and the cleavage of these sialic acids by neuraminidase might interrupt the binding of factor H to host cell membranes.[277, 287] Complement components themselves are glycosylated as well and, for instance, the interactions of factor I with C3b and factor H are regulated in part by these glycans.[288] Removal of the sialic acid group from these glycoproteins by neuraminidase can alter these interactions, thereby dysregulating the complement system. In a patient with a COG7 deficiency, in which the glycosylation of proteins was impaired due to a defect in retrograde vesicular transport of the Golgi apparatus, thrombocytopenia, anemia, and mild proteinuria were seen.[289] Macrothrombycytopenia, hemolytic anemia, and renal failure (most probably due to tubular necrosis) were recently identified in a patient with a CMP-sialic acid transporter defect that results in reduced biosynthesis of sialylated proteins as well.[290] It is not known if the symptoms resembling those in HUS in these congenital disorders of glycosylation (CDG), were caused by deglycosylation of complement proteins. More research is needed to examine the role of desialylated complement proteins in the pathogenesis of SP-HUS.

A few reports on complement activation in SP-HUS patients have been published: C3 levels were low in the acute phase of the disease, but levels normalized within a few days.[91, 222] This indication for complement activation was also shown in Chapter 9 in the SP-HUS patient with autoantibodies against FH: the C3d/C3 ratio depicting complement consumption was increased in the acute phase and normalized to control ranges in remission, as in STEC-HUS and aHUS patients. In our hospital, four other SP-HUS patients were treated in the last decade. Unfortunately, serological complement levels in the acute phase of the disease were only obtained in one of them. This patient also displayed normal C3 and C4 levels, but increased C3d levels. and one of our patients was positive for autoantibodies against FH (Chapter 9), which has not been described before in SP-HUS. No mutations were identified in one of the screened complement genes (*FH*, *FI*, and *CD46* in all patients, extended with *FB* and *C3* in one patient); none of the patients were screened for the presence of αFH.

Although no large cohorts have been screened, the presence of complement mutations might not directly influence the course or outcome of SP-HUS. In a case series of five patients, genetic screening revealed complement aberration in three of them.[91] These three patients, however, had a relatively good outcome with no sequelae or recurrence, while the two remaining patients without mutations died due to severe intracranial hemorrhages during the acute phase of disease. The mortality of SP-HUS in the first episode is high, but the potential disease recurrence in SP-HUS seems

low: about 100 cases have been reported with outcome and follow-up data, but no recurrences have been reported so far.[91] It is not known for how many of these patients genetic workup of the complement system has been performed.

Results of mutational screening of associated HUS genes will most probably not affect therapeutic decision making, but can give more information on the role of the patient's genetic background in the pathogenesis of the disease. Patients with a genetic or acquired complement aberration resulting in a dysregulated complement system might have an altered host response. In Chapter 11, we showed that factor H deficient mice have higher bacterial counts in blood, more disease symptoms, and increased acute injury markers after *Streptococcal pneumoniae* infection. Complement deficiency did not influence the formation of glomerular TMA in mice; this was probably a result of the pneumococcal infection in general. The observed decreased host response, however, may result in increased bacterial load in patients as well, which is associated with increased HUS risk: a high incidence of empyema is seen in SP-HUS cases in several cohorts.[50, 56, 291] Patients with a dysregulated complement system might therefore be more prone to develop HUS after *Streptococcus pneumoniae* infection.

12.4. Future perspectives

This thesis has clearly shown that the complement system is involved in the disease course of HUS secondary to an infection: patients had increased complement activation markers in the acute phase of the disease and mutations and/or autoantibodies against FH were present in STEC-HUS and SP-HUS patients as well. As the complement system is one of first parts of the innate immunity to be activated when micro-organisms invade the human body, we cannot exclude that the observed activation in STEC-HUS and SP-HUS is only an infection-related phenomenon, for example due to produced endotoxins by the bacteria. No study has compared levels of complement activation products in patients with STEC-HUS and patients undergoing infection with *E. coli* whom did not develop HUS, and therefore we cannot exclude that the complement activation is an infection-related phenomenon due to for instance bacterial LPS. A prospective study is needed to investigate this.

12.4.1. Diagnosis of the hemolytic uremic syndrome

Based on clinical presentation, it sometimes is difficult to distinguish between individual STEC-HUS and aHUS patients: no age difference is seen and both STEC-HUS and aHUS present with gastro-intestinal problems, including diarrhea (Chapter 6 and Chapter 9).[93] This again clearly shows that postdiarrheal onset does not exclude the possibility of aHUS or that the absence of diarrhea excludes STEC-HUS. This makes differentiating between etiologies for the general pediatrician, whom not often see HUS patients due to the low incidence rate, sometimes even more difficult and this may result in a delay of adequate treatment in these patients. Measurement of serological alternative pathway activation products, might simplify this process: levels of C3bBbP, C3b/c and the C3d/C3 ratio were all higher in aHUS patients than in STEC-HUS patients. As C3d is a more commonly performed measurement than C3bBbP and C3b/c, the C3d/C3 ratio is preferred, especially because this ratio can be used to monitor disease activity in all HUS forms as well. An international cohort, however, is required to achieve sufficient number of patients and the throughput time of the analyses needs to be improved before this diagnostic tool can be added to the current guidelines in diagnosing and monitoring the hemolytic uremic syndrome.

In case STEC-HUS is suspected, a fecal and a rectal swab culture on specific culture plates (sorbitol-MacConkey agar) and simultaneous testing by PCR and/or EIA for the presence of Stx1 and Stx2 is advised. More importantly, serological investigation for antibodies against LPS of the most common STEC serogroups can accelerate the process of discovering the disease background. In several patients included in the study in Chapter 9, the pathological STEC species could not be serotyped by means of fecal culture, but serological screening within the first days after admission revealed anti-O157 LPS antibodies and specific therapy could be started for STEC-HUS. Retrospective analysis of both fecal and serology tests in 50 STEC-HUS patients performed in our institution revealed an increase in STEC diagnosis of 12% with using serology techniques (unpublished data). Therefore, anti-LPS antibody detection needs to be implemented in standard STEC-HUS diagnosis algorithms. At this moment, it is not known how many STEC-HUS patients are culture-negative, but do possess serological antibodies against STEC-LPS, but a retrospective and prospective study in an (inter)national cohort could answer this question.

Even though literature states that ~40% of the non-STEC-HUS cases is preceded by an pneumococcal infection, we could include only one SP-HUS patient in a time-period of four years (1/11; 9%). The lack of familiarity with SP-HUS by the general pediatrician and other pediatric specialists might have

caused that they have overlooked the diagnosis: the similarity to disseminated intravascular coagulation often seen in sepsis, in which thrombocytopenia, hemolytic anemia, and multiorgan failure are seen as well, make the diagnosis of SP-HUS challenging. Awareness for SP-HUS by the pediatricians therefore remains important.

12.4.2. Monitoring of the hemolytic uremic syndrome

Monitoring of complement activation will become more and more important with the implementation of complement inhibition therapy in aHUS and sophisticated methods are needed as routine methods regularly used in clinical setting (AP/CP activity, C3/C4 levels) can give results similar to healthy controls (see Chapter 6 and Chapter 9). Even though the complement dysregulation in aHUS mostly occurs on the level of the glomerular endothelium, the proposed method to monitor the deposition of C3 and C5b-9 on unstimulated HMVEC is labor intensive and technically complex and can certainly not be performed in every clinic.[98] We performed a comprehensive analysis of complement activation in aHUS patients in the acute phase of disease and in remission to identify new biomarkers in the fluid phase for disease activity that could be measured fast and easy via ELISA. The complement activation markers C3d (especially the C3d/C3 ratio), C3bBbP, C3b/c were increased in the acute phase of disease (see Chapter 8 and Chapter 9), but not in the remission phase. This normalization of alternative pathway activation existed even in patients with a clear complement dysregulation due to genetic aberrations, as shown in Chapter 8. Therefore, these fast complement activation assays could be used in early detection of a relapse of aHUS, especially in patients undergoing complement inhibition therapy. In Chapter 9, we showed that the same activation markers also discriminated between the acute and convalescent phase of STEC-HUS and SP-HUS and may therefore be implicated in monitoring disease activity of HUS of any etiology.

12.4.3. Genetic screening in the hemolytic uremic syndrome

Genetic and acquired complement aberrations patients can influence the outcome of aHUS, the success rate of different treatment options such as renal transplantation and eculizumab administration, and the recurrence risk. As mutations are also identified in STEC-HUS and SP-HUS patients, all HUS patients of any etiology should be screened for complement mutations and autoantibodies against factor H.

So far, mutations have been found in aHUS patients in approximately ten genes encoding proteins of both the complement system and the coagulation pathway, and in several patients, mutations have

been identified in more than one associated gene. A next generation based gene panel that targets all genes involved in the complement system, the coagulation system, and the endothelial glycocalyx simultaneously needs to be implemented in the diagnostic routing, as it can maintain or even improve current diagnostic yields in a more efficient manner. Also functional genomics will become more and more important to unravel the functional consequences of identified genetic aberrations. Predicting the likely functional impact of identified sequence variation can be performed by *in silico* prediction software, but these are not always accurate. Prediction programs like Polyphen-2, which predicts the possible functional effects of human non-synonymous variants, extract annotations that are already present in for instance the dbSNP database of the National Centre of Biotechnology Information.[292] Several established mutations identified in aHUS patients nowadays have dbSNP numbers as they have been identified in next generation sequencing projects. The p.Arg1210Cys variant in *CFH*, which has a frequency in the European population of only 0.03%, is annotated in the dbSNP database (rs121913059) and based on that would not be predicted as pathogenic by prediction software, even though it clearly influences the binding of FH to C3b[152]. For most functional analysis methods that are nowadays available, such as crystallography, Biacore, and ELISAs, recombinant protein production and purification is needed, a time-consuming matter. Preferably, one would isolate the mutant protein from the patient's blood to execute binding assays to individual proteins, but as many mutations are found heterozygously, i.e. on only one allele, isolation is complicated. An assay to analyze the functional activity of the complement convertases and the membrane attack complex separately in patient serum without the need of protein purification has recently been developed.[129] This allows one to study the functional consequences of mutations in a situation in whole serum, the natural environment of complement activation *in vivo*. In the current design, this assay cannot point out the exact effect mechanism of the present mutation, but modifications such as the addition of individual purified proteins or its inhibitors can be used to pin-point the site of action. Standardization of this assay still needs to be performed before it can be used in diagnostics.

12.4.4. Establishment of an independent international registry for hemolytic uremic syndrome

Several HUS registries have been set up in the last decade (i.e. the Austrian registry in Innsbruck, the Oklahoma TTP-HUS registry, and the Italian international registry of recurrent and familial HUS and TTP in Bergamo), but all registries focus on STEC-HUS or aHUS and never on both diseases. Only with an extensive database with both genotype and phenotype, we can further improve our insights into the pathogenesis of the different rare HUS forms, which will improve diagnosis, care, and treatment

of HUS in general. Therefore, an international registry with clinical information on initial presentation, disease course, outcome, long-term follow-up, and diagnostic results (microbiology, serology, and genetics) for every patient diagnosed with HUS (STEC-HUS, SP-HUS, aHUS) of any age is indispensable. This registry preferably remains independent from pharmaceutical companies and is owned by the collaborating clinicians.

Along with this registry, a new proposal for the nomenclature of different HUS subgroups is needed. The current nomenclature used by researchers and clinicians can be confusing. Patients with hemolytic anemia, thrombocytopenia, and acute renal failure are divided in two groups: typical or diarrhea-positive (D^+) HUS due to a STEC infection, and atypical or diarrhea-negative (D^-) HUS. As diarrhea can also be a prodrome in patients with atypical HUS[93], this terminology is not able to distinguish between typical and atypical HUS. Furthermore, aHUS is nowadays mostly used for complement-mediated HUS, but in the current terminology, all non-STEC-HUS etiologies, including SP-HUS and for instance pregnancy-induced HUS, are part of this subdivision. The best way to divide HUS subgroups is based on etiology, i.e. STEC-HUS, SP-HUS, complement-mediated HUS (in which no preceding infection with STEC or SP is seen), pregnancy-induced HUS, and idiopathic HUS for ultra-rare cases associated with viruses, medication, or systemic diseases.

12.4.5. Treatment of the hemolytic uremic syndrome with complement inhibitors

The approval in 2012 of the complement inhibiting monoclonal antibody eculizumab for the treatment of atypical HUS has indicated a new era. Since the first successful reports of eculizumab treatment in aHUS patients, many reports have followed, describing aHUS and STEC-HUS patients that received eculizumab to rescue their native kidneys or to prevent a recurrence after kidney transplantation.[78, 103, 120, 293]

Only one systematic review to determine the efficacy and safety of eculizumab treatment in aHUS patients has been performed, but just small, uncontrolled clinical trials of the pharmaceutical company have been included.[294] No meta-analyses have been published yet. The authors of the systematic review state that further research is needed to evaluate eculizumab, ideally using patient-related clinical outcomes. No such clinical trials are enrolling aHUS patients at this moment; neither is eculizumab mentioned in the Cochrane Library (a database with i.e. systemic reviews and health technology assessments; www.cochrane.org) in relation to aHUS. At the moment, there is no consensus document for the treatment of adult aHUS patients in Europe, although both a French and a Spanish study group recently published a diagnostic algorithm including treatment options for

aHUS.[293, 295] They state that eculizumab treatment should be life-long, but also mention that in some patients groups withdrawal of eculizumab might be an option. In 2009, The European Study Group for atypical HUS published guidelines for the initial assessment and early management of children with aHUS, in which of course eculizumab treatment was not implemented.[96] In the audit analysis of this guideline, the benefits and risks for this therapy in children could not be evaluated, as the results of the prospective pediatric trials with eculizumab for aHUS were not published yet.[296] These results have recently been presented at several international conferences (European Society of Pediatric Nephrology 2014, American Society of Hematology 2014, American Society of Nephrology 2014): it clearly has been shown that early intervention with eculizumab improved hematologic and renal parameters, 82% of the patients discontinued dialysis, and no patient has to start dialysis during the study period of 26 weeks.

The results of the prospective trials make that eculizumab is now recommended as the first-line treatment in both children and adult aHUS patients. Eculizumab, however, is extremely expensive: costs per year vary from €82,000 for a 1-year old child up to €500,000 for an adult aHUS patient (average cost for usual care per patient: €360,000), which makes it difficult to implement in our health care system.

In post-transplantation settings, eculizumab is stated in the published algorithms as first-line therapy in cases of aHUS recurrence.[293, 295] We showed that kidney transplantation in adult patients, even those at high risk for recurrence of disease in the graft due to a *CFH* mutation, can be successful without prophylactic eculizumab.[297] A protocol that minimizes cold ischemia time, reduces the risk of rejection and provides endothelial protection was used in four adult aHUS patients and after 16-21 months of follow-up, none of the patients developed a recurrent aHUS or a rejection.[297]

In recent years, the role of the complement system has become more and more important in not only the hemolytic uremic syndrome, but also in for instance age-related macular degeneration (AMD), ANCA vasculitis, ischemia/reperfusion injury, rheumatoid arthritis, and different transplantation settings.[298] Alexion Pharmaceuticals, the producer of eculizumab (Soliris®), is therefore exploring the use of eculizumab in other, mostly ultra-rare, diseases. In Table 12.1, the diseases for which a clinical trial has been conducted are shown. The most recently reported disease in which eculizumab might be a treatment option is thrombotic microangiopathy associated with

Table 12.1. Clinical trials performed by Alexion Pharmaceuticals to evaluate the use of eculizumab in complement-mediated diseases.[a]

Disease	Status[a]	Result[b]
Paroxysmal nocturnal hemoglobinuria	Completed	FDA approval: Mar 19, 2007; EMA approval: Jun 20, 2007
Atypical hemolytic uremic syndrome	Completed	FDA approval: Sep 23, 2011; EMA approval: Nov 24, 2011
Neuromyelitis optica	Completed	Orphan designation EMA: Aug 5, 2013
Myasthenia gravis	Completed	Orphan designation EMA: Jul 29, 2014
Shiga-toxin related HUS	Completed	Orphan designation EMA: Jul 04, 2012
Age-related macular degeneration	Completed	
Mild allergic asthma	Completed	
Dermatomyositis	Completed	
Kidney graft reperfusion injury	Recruiting	Orphan designation EMA: Mar 26, 2014
Catastrophic antiphospholipid antibody syndrome	Recruiting	
Delayed graft function in kidney transplantation	Recruiting	Orphan designation EMA: Feb 19, 2014
Primary MPGN	Recruiting	
Cardiac transplantation	Enrolling by invitation	
Chronic cold agglutinin disease	Active, not recruiting	
Dense deposit disease and C3 nephropathy	Active, not recruiting	
Complement-mediated injury in kidney transplantation	Active, not recruiting	
Guillain-Barré syndrome	Not yet recruiting	
Antibody-mediated rejection in kidney transplantation	Not yet recruiting	
ANCA vasculitis	Withdrawn	No patients enrolled
Hematopoietic stem cell transplantation-associated TMA	No clinical trial	

[a] www.clinicaltrials.gov
[b] www.fda.org; www.ema.europe.eu

severe hematopoietic stem cell transplantation; however, no clinical trial for eculizumab in HSCT is being conducted.[299] The use of eculizumab in STEC-HUS is still questionable. Even though individual patients can respond rapidly with efficient recovery, no significant differences were seen in larger cohorts on mortality or morbidity with the use of eculizumab in STEC-HUS patients.[46, 47, 119, 120] A randomized controlled trial is needed in which eculizumab is administered at a time point early after diagnosing the patient with STEC-HUS and not only to the most severely ill patients.

Next to eculizumab for PNH and aHUS, only one other complement inhibitor has been approved by the FDA and the EMA for clinical use (recombinant C1 inhibitor for hereditary angioedema). However, many drug candidates are in the pipeline, as depicted in Figure 12.2.[300] As complement dysregulation in HUS occurs at both the alternative and terminal pathway, complement inhibiting strategies at C3 level might be just as specific for aHUS than eculizumab treatment. At this moment, the C3 inhibiting peptide compstatin and its analogs are tested in a clinical phase 2 trial in age-related macular degeneration (AMD) and in preclinical phases in for instance aHUS, PNH, and transplantation.[300] Compstatin can block the activation of C3, thereby blocking C3b opsonization, the amplification loop of the alternative pathway, the activation of the terminal complement pathway, and the formation of the anaphylatoxins C3a and C5a.[301, 302] To prevent the formation of C3 convertases, antibodies against factor B, factor D, properdin, and C3b have been disclosed, but not for clinical trials in aHUS so far.[303-308] The natural complement inhibitors present in the human body are object for therapeutic complement inhibitor development as well. Soluble CR1, which is a cofactor for FI in the regulation of the alternative pathway, has successfully been used in one patient with dense deposit disease, but a longer trial in a larger patient population still has to be performed.[309] Recombinant factor H has been considered, but no clinical trials are being conducted at the moment. The manufacturer of eculizumab, Alexion, is currently evaluating the protein TT30 that combines SCR1-5 of FH with the N-terminal four domains of CR2, which can bind C3 degradation products on sites of ongoing complement activation[310]; TT30 is being tested for the use in PNH in a phase 1 trial. A FH derivate that still has the regulatory and target domains, mini-FH, gives promising results in PNH models.[311] Peptides that can recruit and bind host factor H are being evaluated as well.[75] As the incidence rate of HUS is very low (1 in 2 million for aHUS), trials to investigate new complement inhibitors are very expensive and multi-centre, multi-national cohorts are needed. A international, independent registry could facilitate these trials.

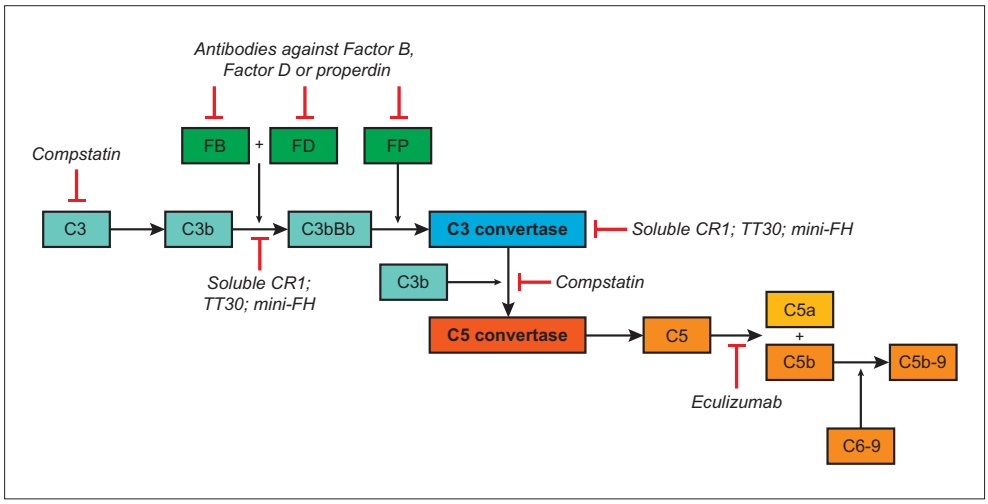


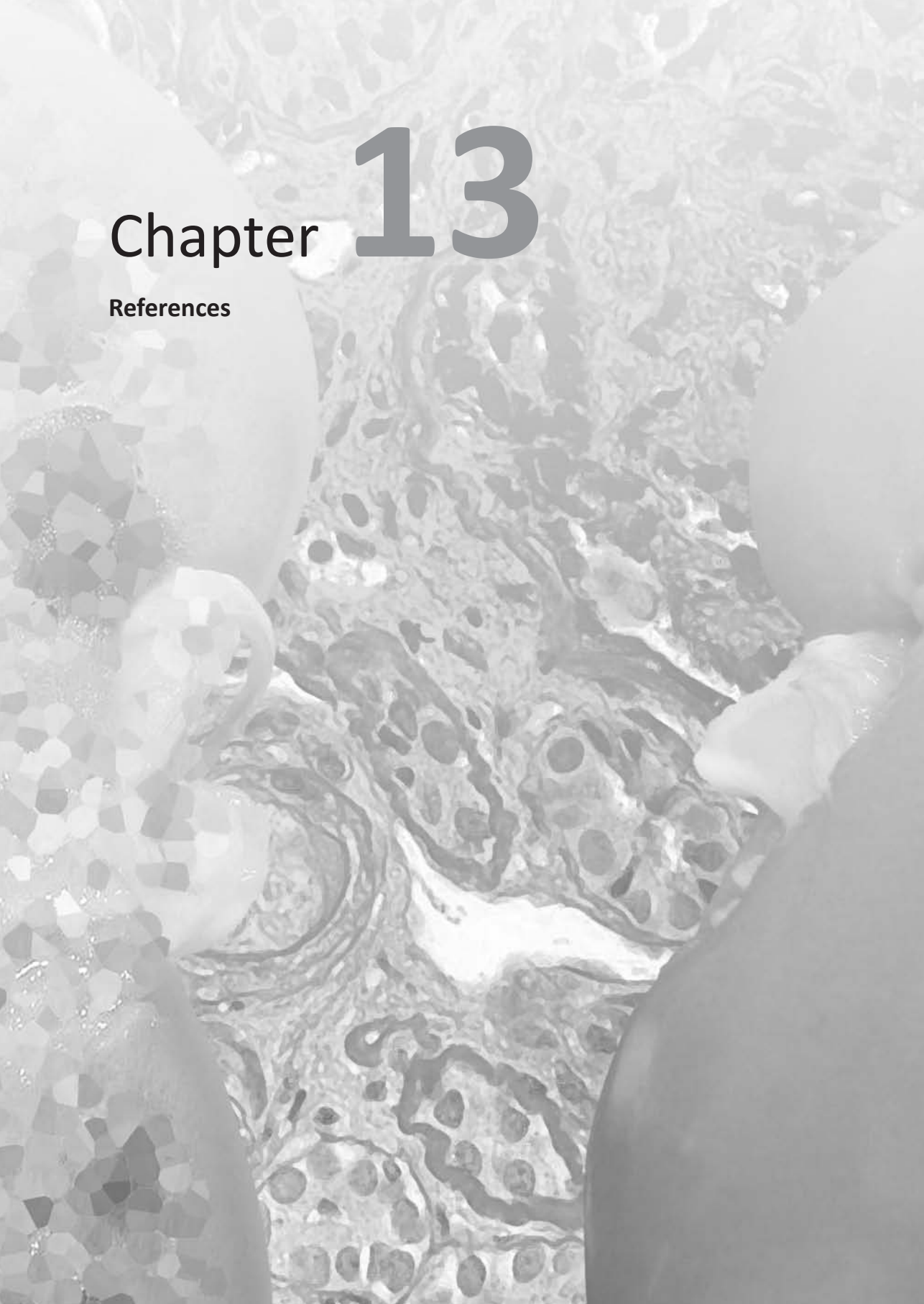
Figure 12.2. Schematic overview of the alternative and terminal pathway of the complement system. The central complement component C3 is spontaneously activated at very low rate to form C3b, which is able to attach to the surfaces of pathogens and host cells. There it binds complement factor B (FB), which is proteolytically activated by Factor D (FD). The resulting C3bBb complex is stabilized by properdin (FP) and this C3 convertase can cleave and activate more C3 molecules. This activation leads to amplification of the complement cascade via the C5 convertase, to the formation of the membrane attack complex (C5b-9), and, eventually, to cell lysis. The complement inhibiting therapeutics acting on the level of C3 and C5 that are currently approved or in development are depicted at the level of action.

As long as no other complement inhibiting therapeutics are on the market, eculizumab remains the best option in aHUS patients. Due to the extreme costs of eculizumab, however, the treatment is not readily available for all patients. One way to optimize cost-efficacy involved in the treatment is by adapting the treatment to the individual. Recently reported and preliminary data indeed indicate that we may be exceeding the pharmacodynamic target and that even despite the severity of disease, we may need less drug for effective treatment in some patients. The dose regimen used in the trials for registration of eculizumab in aHUS were targeted at eculizumab trough levels above 50-100 ug/ml to ensure complete complement blockade. Pharmacokinetic data in the trials revealed levels of eculizumab ranging between 93-342 µg/ml and 113-431 µg/ml in adults and 104-392 and 109-414 µg/ml in adolescents, and thus more than twice as high as reported by EMA to achieve a complement inhibition.[104] Furthermore, different experiences show that increased intervals between doses, or even therapy discontinuation, is possible in some patients without reactivation.[312] Ardissino *et al.* recently reported the discontinuation of eculizumab treatment in 10 aHUS patients. Seven patients did not experience a recurrence of disease; the remaining three patients had a recurrence of disease within 6 weeks after the treatment was stopped.[313]

Although eculizumab certainly has changed the future perspectives of patients with aHUS, many unsolved questions remain: who should receive the drug, what treatment schedules should be used, and how long should therapy be continued. As proven by several case reports, it might possible to settle with less amount of drug or even stop the treatment after a certain amount of time in more patients. The relationship between pharmacokinetics and pharmacodynamics and tailored therapy needs to be demonstrated in a robust and controlled fashion. Adapting dosage schemes by means of therapeutic drug monitoring based on eculizumab levels and for instance the alternative pathway biomarkers investigated in this thesis, will improve the quality of live for patients and their family members, as the burden of traveling to the hospital every two weeks for eculizumab therapy is very high. Furthermore, as therapeutic drug monitoring can possibly result in a 50% reduction of the amount of drug to be used and thus a saving of €180,000 for an average patient, this will result in an extreme cost reduction of at least €1,5 million a year in the Netherlands. The Netherlands Organisation for Health Research and Development (ZonMw) has recently granted this study in their 'Goed Geneesmiddelen Gebruik' program.

12.5. Concluding remarks

We have demonstrated that the complement system is involved in all forms of the hemolytic uremic syndrome. In both infection-induced HUS and aHUS patients, the complement system is activated in the acute phase of the disease and not in the convalescent phase. Measuring alternative pathway activation products, in particular the C3d/C3 ratio, might help in motoring disease activity and in distinguishing between the different HUS etiologies. Interestingly, genetic and/or acquired complement aberrations (mutations and/or autoantibodies) were not only identified in aHUS patients, but also in STEC-HUS and SP-HUS. This indicates that genetic screening is advised in these patients as well. The exact role of altered complement activation in the pathogenesis of STEC-HUS and SP-HUS has not been fully elucidated with this project and more research is needed before complement inhibiting therapeutics may be included in the treatment guidelines of infection-induced HUS.



Chapter 13

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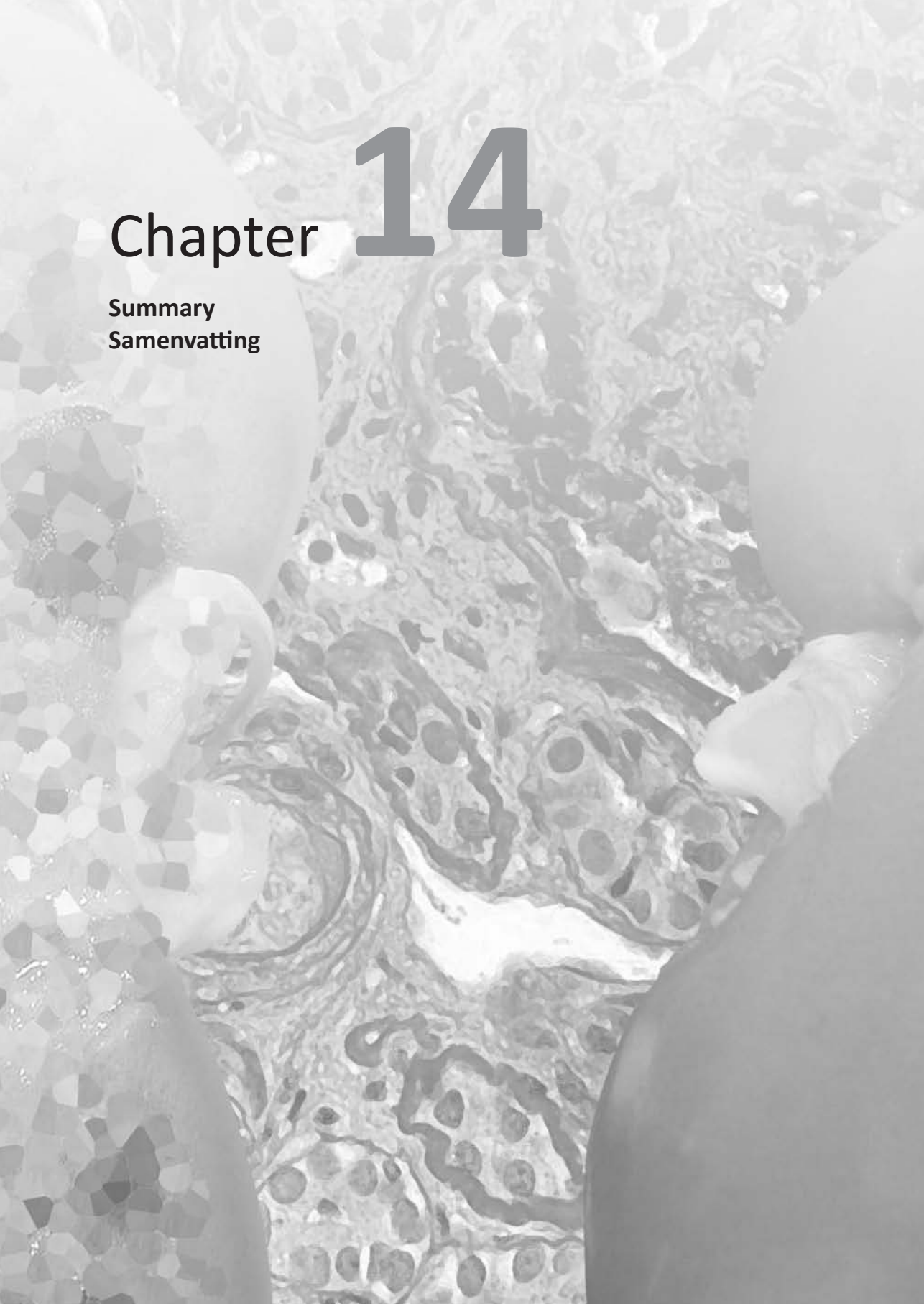

Chapter 14

Summary
Samenvatting

Summary

The hemolytic uremic syndrome (HUS) is a rare and severe thrombotic microangiopathy characterized by hemolytic anemia, thrombocytopenia, and acute renal failure. In more than 90% of the cases in childhood, the disease is triggered by an infection with Shiga-like toxin producing *Escherichia coli* (STEC). The most important causes seen in the remaining 5-10% of the HUS cases are dysregulation of the alternative pathway of the complement system due to genetic mutations (here called atypical HUS or aHUS) and an non-enteric infection with *Streptococcus pneumoniae* (SP-HUS). The complement system is part of the innate immunity. It induces cell lysis by the incorporation of the membrane attack complex and activates and attracts leukocytes. The complement system, however, does not discriminate between pathogens and host cells. To protect our own cells the system is therefore tightly regulated on both the cell surface and in the fluid phase. Non-host surfaces that lack these regulators are attacked and damaged by the complement system. A mutation in one of the regulators will lead to uncontrolled activation of the complement system on host cells resulting in cell damage, especially to those in the microcirculation of the kidney. These mutations, mostly seen in the alternative pathway of the complement system, are described in ~50-60% of the aHUS patients.

It has been shown that both STEC and *S. pneumoniae* can protect themselves against complement activation. This knowledge, the fact that dysregulation of the alternative complement pathway due to mutations is believed to play an important role in the pathogenesis of aHUS, and the similarity of the clinical manifestations, lead to the aim of this thesis: to study and define the role of the complement system in three forms of HUS (STEC-HUS, SP-HUS, and aHUS).

The first part if this thesis is focused on genetic studies in aHUS patients as the presence of mutations can be of prognostic value for the outcome of disease and for treatment options. For instance, patients with a mutation in the *CFH* or *CFI* gene have a worse outcome after renal transplantation (recurrence: 80-100%) than patients with a *CD46* mutation (recurrence 0–20%). In **Chapter 2**, **Chapter 3**, **Chapter 4**, and **Chapter 6**, we screened a group of 70 aHUS patients for mutations in genes encoding associated complement (regulating) proteins. In just over 50% of these patients, a potential disease causing genetic aberrations or the presence of autoantibodies against the key regulator Factor H was identified. In the remaining patients, however, aHUS could not be explained by a mutation in any of the screened genes. Genetic screening of all associated genes via regular methods (PCR and Sanger sequencing) is labor intensive, costly, and time consuming. The

application of next generation sequencing might be an attractive alternative routing for mutation scanning in aHUS patients, but in **Chapter 5** we have shown that the mutation hotspot in *CFH* is not fully covered with the current whole exome sequencing technique. Therefore, at this moment next generation sequencing can only be applied when combined with conventional amplicon based (Sanger) sequencing for the missing *CFH* region and the deep intronic SNPs of aHUS associated haplotypes. Genetic or acquired complement aberrations are not only present in aHUS patients, but also in more than 30% (8/26) of the investigated STEC-HUS and SP-HUS patients in **Chapter 9**. This high mutation rate needs to be confirmed in other large patient cohorts and detailed clinical follow-up data are needed to determine the influence of these mutation on disease course and outcome in STEC-HUS and SP-HUS. Based on these results, it is advised to not only screen aHUS patients for complement aberrations, but also STEC-HUS and SP-HUS patients. In that way, the knowledge on their role in pathology can be further extended.

The therapeutic management of HUS can be very difficult, especially when other diseases are present in the patient. This is shown in **Chapter 7**, where a patient is described with both STEC-HUS and hemophilia A. The administration of recombinant coagulation factor VIII was needed to control hemorrhagic colitis and to prevent severe neurological complication, but might have enhanced to thrombotic microangiopathic process.

The second part of the thesis is focused on biomarkers for complement activation in HUS patients, on complement evasion by STEC O157:H7, and on host response due to complement dysregulation. Functional analysis of serological complement levels in HUS patients remains very limited. We therefore performed a comprehensive study of complement activation in both the acute and convalescent phase of the disease, first in aHUS patients (**Chapter 8**) and later in STEC-HUS and SP-HUS patients as well (**Chapter 9**). We demonstrated that the alternative pathway activation markers C3d, C3bBbP, and C3b/c were increased in all patient groups in the acute phase, but not in remission. The C3d/C3 ratio gives the best discrepancy between the acute and convalescent phase and could therefore be used as a marker to monitor disease activity in HUS of any etiology. Furthermore, all above-mentioned alternative pathway activation products were significantly more increased in the acute phase of disease in aHUS patients than in STEC-HUS patients and may be used as a biomarker to distinguish at admission between these two diseases. We could not link any of ten STEC O157:H7 proteins investigated in **Chapter 10** to the identified complement activation seen in STEC-HUS patients; nor could we link the streptococcal proteins neuraminidase A, pneumolysin or PspC to the development of thrombotic microangiopathy in mice (**Chapter 11**). We did, however,

observe a decreased host response in Factor H deficient mice that lead to a higher bacterial load. In patients, this is associated with increased risk to develop HUS, and patients with a dysregulated complement system due to genetic or acquired aberrations might therefore be more prone to develop SP-HUS.

The complement inhibitor eculizumab has certainly changed the future perspectives of patients with aHUS, but many unsolved questions remain: who should receive the drug, which optimal treatment schedules should be used, and how long should therapy be continued. It is even undecided if prophylactic treatment is needed in transplantation settings. As eculizumab is very expensive (up to €500,000 per treatment year), cost effectiveness in both standard and new treatment algorithms should be evaluated in carefully conducted prospective cohort studies. For this, new complement assays and biomarkers will be obligatory.
An important role of the complement system in the pathogenesis of infection-induced HUS has not been fully verified so far, and more research is needed before complement inhibitors are implemented as treatment options in these HUS etiologies.

In this thesis, we aimed to further elucidate the role of the complement system in the pathogenesis of STEC-HUS, SP-HUS, and aHUS. In **Chapter 12**, we discuss the implications of the results. Investigation in a larger cohort is needed, but based on our results, the alternative pathway activation markers C3bBbP, C3b/c, and in particular the C3d/C3 ratio may be used to monitor disease activity and to distinguish between different HUS etiologies at time of presentation. As we also identified genetic and/or acquired complement aberrations in patients with both complement-mediated and infection-related HUS, genetic screening is not only advised in aHUS, but in STEC-HUS and SP-HUS as well. The establishment of an independent international registry for HUS with both clinical information and diagnostic results (microbiology, serology, and genetics) will further improve our insights into the pathogenesis of the different HUS forms.

Nederlandse samenvatting

Het hemolytisch uremisch syndroom (HUS) is een zeldzame, maar ernstige nierziekte. De ziekte wordt gekarakteriseerd door hemolytische anemie (een abnormale afbraak van rode bloedcellen), trombocytopenie (een tekort aan bloedplaatjes) en acuut nierfalen. In meer dan 90% van de gevallen op kinderleeftijd wordt de ziekte voorafgegaan door een infectie met de Shiga-like toxine producerende *Escherichia coli* (STEC) bacterie. De belangrijkste oorzaken in de overige 5-10% zijn een verkeerde regulatie van het complementsysteem door genetische afwijkingen (hier atypisch HUS of aHUS genoemd) en een infectie met de *Streptococcus pneumoniae* bacterie (SP-HUS). Het complementsysteem is onderdeel van het aangeboren immuunsysteem en zorgt voor de eerste afweer tegen indringers. Het induceert cellysis door het inbouwen van het membrane attack complex in het celmembraan van bijv. bacteriën en het activeert leukocyten om te infectie op te ruimen. Het complementsysteem ziet echter geen verschil tussen indringers en eigen cellen. Om de eigen cellen te beschermen wordt het complementsysteem daarom strikt gereguleerd op zowel het celoppervlak als in de bloedbaan. Indringers die deze beschermmechanismen niet hebben, worden aangevallen en beschadigd. Een genetische afwijking of mutatie in één van de regulatoren zal leiden tot een ongecontroleerde activatie van het complementsysteem op eigen cellen met celschade als gevolg, vooral aan de cellen in de microcirculatie van de nieren. Deze mutaties, die meestal worden gevonden in genen die coderen voor onderdelen van de alternatieve route van het complementsysteem, worden beschreven in ~50-60% van de aHUS patiënten.
Het is bekend dat zowel STEC als *S. pneumonaie* zichzelf kunnen beschermen tegen complementactivatie. Deze wetenschap, het feit dat een dysregulatie van de alternatieve route van het complementsysteem een belangrijke rol lijkt te spelen in de pathogenese van aHUS, en de overeenkomsten in de klinische symptomen heeft geleid tot het doel van dit proefschrift: het verder ontrafelen van de rol van het complementsysteem in de drie verschillende vormen van HUS (STEC-HUS, SP-HUS en aHUS).

In het eerste deel van dit proefschrift worden de genetische studies in aHUS patiënten beschreven. De aanwezigheid van mutaties kan van invloed zijn op het ziektebeloop en op de verschillende behandelopties. Patiënten met een mutatie in *CFH* of *CFI* hebben bijvoorbeeld een hoge kans op terugkeer van ziekte na een niertransplantatie (80-100%), terwijl in patiënten met alleen een *CD46* mutatie die kans 0-20% is. In **Hoofdstuk 2**, **Hoofdstuk 3**, **Hoofdstuk 4** en **Hoofdstuk 6** hebben we een groep van 70 aHUS patiënten gescreend op de aanwezigheid van mutaties in genen die coderen

voor complementeiwitten en geassocieerd zijn met de ziekte. In iets meer dan 50% van de patiënten hebben we een mogelijke ziekteveroorzakende verandering of de aanwezigheid van autoantilichamen tegen Factor H, de belangrijkste regulator van de alternatieve route, gevonden. In de overige patiënten is echter geen mutatie gevonden in één van de gescreende genen die het ontstaan van aHUS kan verklaren. De genetische screening van alle geassocieerde genen via reguliere methoden (PCR en Sanger scequencing) is arbeidsintensief, kostbaar en tijdrovend. Het toepassen van next generation sequencing technieken zou daarom een mogelijk interessante alternatieve manier zijn om aHUS patiënten te screenen op mutaties. In **Hoofdstuk 5** hebben wij echter laten zien dat de hotspot voor mutaties in *CFH* niet volledig wordt gedekt met de huidige whole exome technieken, waardoor in 7,5% van de patiënten de mutatie wordt gemist. Daarom kan op dit moment next generation sequencing alleen worden toegepast wanneer het wordt gecombineerd met traditionele Sanger sequencing voor de niet-gedekte *CFH* gebieden en voor diep-intronische SNPs in haplotypes geassocieerd met aHUS. Genetische en verworven afwijkingen, zoals de autoantilichamen tegen Factor H, in het complementsysteem zijn niet alleen aanwezig in aHUS patiënten, maar ook in meer dan 30% (8/26) van de onderzochte STEC-HUS en SP-HUS patiënten in **Hoofdstuk 9**. Dit hoge percentage mutaties moet bevestigd worden in grotere patiëntcohorten en gedetailleerde data over klinische follow-up zijn nodig om de invloed van deze mutaties op het ziekteverloop en de gevolgen in STEC-HUS en SP-HUS te bepalen. Gebaseerd op onze resultaten wordt aangeraden niet alleen aHUS patiënten te screenen op complementafwijkingen, maar ook STEC-HUS en SP-HUS patiënten, om zo de kennis over de rol van mutaties in de pathogenese van de ziekte te vergroten.

De behandeling van HUS patiënten kan erg uitdagend zijn, met name wanneer de patiënt ook nog andere ziektes heeft. Dit wordt duidelijk in **Hoofdstuk 7** waar een patiënt wordt beschreven met zowel STEC-HUS als hemofilie A. De toediening van recombinant factor VIII om hemorrhagische colitis onder controle te krijgen en ernstige neurologische complicaties te voorkomen, zou het thrombotisch microangiopathisch proces van de HUS hebben kunnen bevorderd.

Het tweede deel van dit proefschrift is gefocust op het vinden van biomarkers voor complementactivteit in HUS patiënten, op complementevasie door STEC O157:H7 en op de gastheerresponse bij complementdysregulatie. De mogelijkheid tot het meten van complementeiwitten en de complementactiviteit in het bloed van HUS patiënten is beperkt. Daarom hebben wij de complementactiviteit uitgebreid bestudeerd in de acute fase en de herstelfase van de ziekte, eerst aHUS patiënten (**Hoofdstuk 8**) en later ook in STEC-HUS en SP-HUS patiënten

(**Hoofdstuk 9**). Wij hebben laten zien dan de activatieproducten van de alternatieve route (C3d, C3bBbP en C3b/c) verhoogd waren in alle patiëntengroepen in de acute fase, maar niet in remissie. De C3d/C3 ratio geeft het duidelijkst verschil tussen de acute fase en de herstelfase en kan daardoor mogelijk gebruikt worden als een biomarker voor ziekteactiviteit in alle HUS vormen. Verder waren alle activatieproducten in de acute fase significant meer verhoogd in aHUS patiënten dan in STEC-HUS patiënten en kunnen deze gebruikt worden als mogelijk toekomstige biomarker om bij opname onderscheid te maken tussen deze twee etiologiën. De gevonden complementactivatie in STEC-HUS patiënten kon niet verklaard worden door verandering in complementregulatie door één van de tien STEC O157:H7 eiwitten die zijn bestudeerd in **Hoofdstuk 10**. Ook konden we streptokokkeneiwitten neuraminidase A, pneumolysine of PspC niet linken aan het ontstaan van thrombotische microangiopatie in muizen (**Hoofdstuk 11**). We zagen echter wel een veranderde afweer tegen *Streptococcus pneumoniae* in muizen deficiënt voor Factor H wat resulteerde in een meer bacteriën in de bloedbaan. In patiënten is deze zogenoemde hogere bacterieload geassocieerd met een hoger risico op het ontwikkelen van HUS. Patiënten met een verstoorde complementregulatie door genetische of verworven afwijkingen zouden daardoor mogelijk vatbaarder zijn voor het ontwikkelen van SP-HUS.

De behandeling met de complementremmer eculizumab heeft de toekomst van aHUS patiënten zeker veranderd, maar er zijn nog steeds veel onbeantwoorde vragen. Welke patiënten moeten het medicijn ontvangen of kunnen zij ook met andere methodes behandeld worden; wat is het beste behandelschema; moet het medicijn inderdaad levenslang gegeven worden, zoals de farmaceut zegt, of kan er ook afgebouwd worden; moeten patiënten die een niertransplantatie ondergaan inderdaad altijd rond de transplantatie eculizumab ontvangen? Om bovenstaande vragen te kunnen beantwoorden en omdat eculizumab een erg kostbaar medicijn is (tot €500.000,- per behandeljaar), moet het huidige behandelschema vergeleken worden met nieuwe schema's in een prospectieve cohortstudie. Hiervoor zijn nieuwe complement assays en biomarkers essentieel.
Met de resultaten uit dit proefschrift is de exacte rol van het complementsysteem in de pathogenese van infectiegerelateerde HUS niet bevestigd. Voordat complementremmers ook een rol krijgen in de behandeling van deze HUS vormen is eerst nog meer onderzoek nodig.

In dit proefschrift hebben we geprobeerd de rol van het complementsysteem in de pathogenese van STEC-HUS, SP-HUS en aHUS verder te ontrafelen. In **Hoofdstuk 12** bediscussiëren we de consequenties van de resultaten. De activatieproducten van de alternatieve route van het

complementsysteem C3bBbP, C3b/c en vooral de C3d/C3 ratio zouden als biomarkers gebruikt kunnen worden om ziekteactiviteit te monitoren en om onderscheid te maken tussen verschillende oorzaken bij opname van de patiënten, maar dit resultaat moet bevestigd worden in een groter cohort. Het screenen naar afwijkingen in het complementsysteem, zowel genetische als verworven, wordt niet alleen aangeraden in aHUS patiënten, maar ook in STEC-HUS en SP-HUS, aangezien wij ook in die groepen afwijkingen hebben gevonden. Door een onafhankelijke internationale database op te zetten voor HUS waarin zowel klinische informatie en diagnostische resultaten (op microbiologisch, serologisch en genetisch niveau) worden verzameld, zullen de inzichten wat betreft de pathogenese van de verschillende HUS vormen vergroot worden.

Chapter 15

Dankwoord
Curriculum vitae
List of publications
List of abbreviations

Dankwoord

Eindelijk kan ik dan aan het dankwoord beginnen: het meest gelezen, en dus misschien wel het belangrijkste hoofdstuk van mijn proefschrift. Ik wil iedereen danken die op enige wijze betrokken is geweest bij de totstandkoming van dit boekje, op inhoudelijk gebied, met praktische zaken, of door gewoon aanwezig te zijn in de afgelopen jaren. Een aantal mensen wil ik graag in het bijzonder bedanken.

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Dan mijn eerste co-promotor, dr. Nicole van de Kar. Lieve Nicole, wat ben ik blij dat ik jouw eerste promovendus mag zijn! Nog steeds vind ik het verwonderlijk hoe stellig jij aan het eind van mijn bachelorstage kon zeggen dat ik de juiste kandidaat zou zijn voor een promotie-onderzoek binnen de HUS groep, terwijl ik eerst nog twee jaar zou moeten studeren. En zie hier het resultaat van die stelligheid! Ik zal al onze uitstapjes naar de vele congressen in binnen- en buitenland nooit vergeten: na een nacht in het heerlijk slapende prinsessenbed van Janne 8 uur in de auto om naar Jena te gaan; vliegen naar Innsbruck vanaf Frankfurt, maar het vliegtuig missen omdat het verkeerde vliegveld ingesteld stond op de TomTom; al dansend in het Kasnapolsky het succes van VTEC2012 vieren; enz, enz. Ook dank voor al je persoonlijke belangstelling, het meedenken over mijn toekomst, en de soms zelfs nachtelijk Whatsapp-gesprekken!

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Beste paranimfen, lieve Thatjana en Inge. Wat ben ik blij dat jullie achter mij willen staan met mijn promotie! Thatjana, al sinds die eerste zondag van de introductie in het Kronenburgerpark was er een band. Tijdens de studie zijn we elkaar helaas een beetje uit het oog verloren, maar wat was het leuk om mijn mentorzusje opeens weer op het lab tegen te komen. Meteen was het alsof we elkaar niet al een aantal jaar niet hadden gezien en toen we ook nog eens tegelijkertijd zwanger waren, werd het alleen maar leuker. Nu ook nog eens samen bij Genetica, volgens mij komen we niet meer van elkaar af, gelukkig maar! Inge, wat hebben wij al veel gedeeld de afgelopen 12 jaar: hockey tot en met, een geweldig bestuursjaar, vele etentjes, de nodige biertjes, wijntjes en tegenwoordig kopjes thee, stapavonden, sleep-overs in Nijmegen of Vught, goede gesprekken, zwangerschappen en nog veel meer. Dank je wel daarvoor en het leek me niet meer dan logisch om dit moment ook met jou te delen!

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vandaan! Roel Kurvers en Kioa, heel veel succes met jullie onderzoek naar complementgemediëerde glomerulaire ziekten: het is goed om ook weer eens klinisch onderzoekers in de groep te hebben!

Dan de mensen op het lab. Moet ik jullie nu LKN, LGEM of TML noemen, ik weet het niet meer met al die namen die de revue zijn gepasseerd… Dank voor alle gezelligheid op het lab, tijdens de koffiepauzes, bij de TGIFs, de dagjes uit, kortom: in de afgelopen zoveel jaar. Jullie dachten misschien nooit meer van me af te komen en ik vind het ook erg jammer dat ik dit alles nu moet missen. Gelukkig is de koffiekamer dichtbij!

Uiteraard moeten er ook wat mensen van het lab speciaal in het zonnetje worden gezet. Allereerst de analisten van de HUS groep: Thea, Annelies, Annemieke en Joop. Thea, dank je wel voor al je gezelligheid, gezang en kritische noten op het lab. ELISA's draaien zoals jij kan volgens mij niemand! Annelies, je bent altijd in voor een gesprekje over van alles en nog wat en dat was altijd fijn als ik even niet met werk bezig wilde zijn. Annemieke, ook al was je er niet lang, jouw gedrevenheid heeft diepe indruk op me gemaakt! Joop, dank je wel voor die laatste ELISA's die je nog op het eind voor mij wilde draaien. Met jou in de groep maak ik me totaal geen zorgen over alle diagnostische assays die opgezet en geïmplementeerd moeten worden. Verder moet de DNA groep ook even genoemd worden, vooral Roel en Inge. Roel, dank je wel dat jij mij 8 jaar geleden hebt leren pipetteren en sequencen. Ik denk dat ik zonder jouw kennis niet was gekomen tot waar ik nu sta. Inge, bedankt voor al die DNAtjes die je hebt geïsoleerd en voor je altijd snelle antwoord als ik weer eens een vraag moest doorspelen uit de kliniek kwam over welk gen er nu wel of niet gerund was.

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Curriculum Vitae

Dineke Westra werd geboren op 10 januari 1985 te Apeldoorn. In 2002 behaalde zij haar VWO-diploma aan het Bonhoeffer College in Enschede. Na een High School jaar te Alberton, Zuid-Afrika, begon zij in 2003 met de studie Biomedische Wetenschappen aan de Radboud Universiteit Nijmegen. Tijdens haar bachelor liep zij stage bij HUS-onderzoeksgroep van de afdeling Kindergeneeskunde van het Radboudumc in Nijmegen, onder leiding van prof. dr. L.P. van den Heuvel en dr. N.C.A.J. van de Kar, alwaar zij onderzoek deed naar genetische afwijkingen in het gen coderend voor factor H in patiënten met het hemolytisch uremisch syndroom. Het verslag van deze stage werd in 2007 genomineerd voor de dr. Bexprijs voor beste bachelorscriptie Biomedische Wetenschappen. Ze vervolgde hierna haar studie met de master Pathobiologie. Tijdens haar korte masterstage verrichtte zij onder leiding van prof. dr. F.H. van der Westhuizen onderzoek bij het Mitochondrial Research Laboratory van de North-West University in Potchefstroom, Zuid-Afrika. Tijdens haar laatste onderzoeksstage, welke zij wederom liep bij de HUS-onderzoeksgroep van de afdeling Kindergeneeskunde van het Radboudumc in Nijmegen onder leiding van prof. dr. L.P. van den Heuvel en dr. N.C.A.J. van de Kar, werd de basis gelegd voor het promotieonderzoek beschreven in dit proefschrift. Naast haar studie was Dineke werkzaam als student-assistent bij de HUS-onderzoeksgroep, waarvoor zij het Sengers Stipendium van de Stichting Kindergeneeskunde van het Radboudumc en een tweetal Kolff Student Onderzoeker beurzen van de Nierstichting heeft ontvangen. Daarnaast was zij actief in verschillende activiteitencommissies en in het bestuur van de Nijmeegse Studenten Hockeyclub Apeliotes. Na het behalen van haar masterdiploma in 2009 startte ze haar promotieonderzoek op de afdeling Kindernefrologie van het Radboudumc te Nijmegen naar de rol van het complementsysteem in de pathogenese van het hemolytisch uremisch syndroom onder leiding van prof. dr. L.P. van den Heuvel, dr. N.C.A.J. van de Kar en dr. E.B. Volokhina. Dit onderzoek heeft uiteindelijk geresulteerd in meerdere artikelen en dit proefschrift. Tevens heeft zij tijdens haar promotietraject verschillende bachelorstudenten begeleid. De resultaten van het promotieonderzoek zijn gepresenteerd op verschillende nationale en internationale congressen en in 2011 ontving Dineke een award voor de beste poster tijdens de 3rd International Conference – HUS, MPGN & related diseases in Innsbruck, Oostenrijk, en de derde prijs voor de beste presentatie tijdens het najaarssymposium van de Nederlandse Federatie voor Nefrologie.

Dineke is momenteel in opleiding tot laboratoriumspecialist klinische genetica bij de afdeling Genetica van het Radboudumc in Nijmegen. Zij is getrouwd met Gerrit en is moeder van Margje.

List of publications

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List of abbreviations

αFH	autoantibodies against FH
ΔCFHR1/3	homozygous polymorphic deletion of *CFHR1* and *CFHR3*
AMD	age-related macular degeneration
aHUS	atypical HUS
AKI	acute kidney injury
AP	activity of the alternative complement pathway
AP50	activity of the alternative complement pathway
C3	complement component 3
C3	gene encoding complement component 3
C3bBbP	alternative pathway C3 convertase
CAU/ml	complement activation units per ml
cblC	cobalamin C
CD46	membrane co-factor protein
CD46	gene encoding membrane co-factor protein
CDG	congenital disorders of glycosylation
CFB	gene encoding complement factor B
CFH	gene encoding complement factor H
CFHR	gene encoding complement factor H related protein
CFI	gene encoding complement factor I
CFU	colony forming units
CH50	activity of the classical complement pathway
CI	confidence interval
CNV	copy number variation
CP	activity of the classical complement pathway
CRP	C-reactive protein
CT	computed tomography
CVVH	continuous veno-venous hemofiltration
DDD	dense deposit disease
DGKE	gene encoding diacylglycerol kinase ε
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
EspP	*Escherichia coli* secreted protein P
ESRD	end stage renal disease
EVS	Exome Variant Server
FACS	fluorescent-activated cell sorting
FB	complement factor B
FD	complement factor D
FH	complement factor H
FHR	complement factor H related protein
FI	complement factor I
FVIII	factor VIII
Gb_3	globotriaoceramide receptor
GFR	glomerular filtration rate
hB	hemoglobin
HELLP	hemolysis, elevated liver enzymes, and low platelets
HMVEC	human microvascular endothelial cells
HPSG2	gene encoding heparan sulphate
HSCT	hematopoietic stem cell transplantation

HUS	hemolytic uremic syndrome
ICS#2	international complement standard#2
IEF	isoelectric focussing
IL-6	interleukin 6
IPD	invasive pneumococcal disease
IPTG	isopropyl-β-D-thiogalactopyranoside
KIM-1	kidney injury marker 1
LB medium	Luria-Bertani medium
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MAC	membrane attack complex
MCP	membrane co-factor protein
MIP-2	macrophage inflammatory protein 2
MMACHC	gene encoding cobalamin C
NanA	neuraminidase A
NGAL	neutrophil gelatinase-associated lipocalin
NGS	next generation sequencing
NHP	normal human pooled serum
NHS	normal human serum
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	plasma exchange
PF	plasmapheresis
PLG	gene encoding plasminogen
Ply	pneumolysin
PNH	paroxysmal nocturnal hemoglobinuria
PspC	pneumococcal surface protein C
PVC7	7-valent pneumococcal conjugate va
PVOD	pulmonary veno-occlusive disease
rFVIII	recombinant factor VIII
sC5b-9	fluid phase TCC or MAC
SCR	short consensus repeat
SCRC domain	scavenger recptor cysteine rich domain
SIFT	Sorting Intolerant From Tolerant
SNP	single nucleotide polymorphism
SP	*Streptococcus pneumoniae*
SP domain	serine protease domain
SP-HUS	*Streptococcus pneumoniae*-induced HUS
STEC	shiga-like toxin producing *Escherichia coli*
STEC-HUS	STEC-induced HUS
Stx	Shiga-like toxin
T-antigen	Thomson Friedenreich cryptantigen
TCC	terminal complement complex
THBD	gene encoding thrombomodulin
TMA	thrombotic microangiopathy
TTP	thrombocytopenic purpura
TTSS	type III secretion system
WES	whole exome sequencing